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The International Isocyanate Institute (III) on behalf of its members (BASF Corp.; Bayer Corp.; The Dow Chemical Company; ICI Americas, Inc.; and ARCO Chemical) hereby notifies the EPA of the following:

Thesis research report.	"Toluene Diisocyanate-Induced Occupational Asthma"
Name of chemical substance:	toluene diisocyanate benzene, 1,3-diisocyanatomethyl
Chemical Abstracts Service Number	26471-62-5
Name and address of testing organiza	Heleen Scheerens Dept. of Pharmacology and Pathophysiology University of Utrecht The Netherlands

The studies were supported by The National Institute of Public Health and the Environment, Bilthoven, The Netherlands. A copy of the thesis is enclosed.

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Toluene Diisocyanate-Induced Occupational Asthma

Heleen Scheerens

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Toluene Diisocyanate-Induced Occupational Asthma

Tolueendiisocyanaat geïnduceerde beroepsastma

(met een samenvatting in het nederlands)

Proefschrift

ter verkrijging van de graad van doctor
aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. Dr J.A. van Ginkel
ingevolge het besluit van het College van Decanen
in het openbaar te verdedigen
op donderdag 12 juni 1997 des namiddags te 12.45 uur

door

Heleen Scheerens

geboren op 11 December 1969, te Amsterdam

Promotor: Prof. Dr F.P. Nijkamp

Co-promotores: Dr T. L. Buckley
Dr H. Van Loveren

The studies presented in this thesis were performed at the Department of Pharmacology and Pathophysiology, Utrecht University and the Department of Pathology and Immunobiology, National Institute of Public Health and the Environment, Bilthoven, The Netherlands.
The studies presented in this thesis were supported by a special grant from the National Institute of Public Health and the Environment to stimulate cooperation with academia.

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Contents

CHAPTER 1

General introduction

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1 Asthma

Asthma patients can roughly be divided into two groups. In most cases, asthma is associated with atopy, particularly in children. Atopic asthma, also called extrinsic or allergic asthma, refers to the genetic predisposition of individuals to synthesize immunoglobulin E (IgE) specific for certain allergens. The second group, the non-atopic asthmatics, are referred to as 'intrinsic' and this form of asthma is thought to be important in most cases of occupational asthma. In intrinsic asthma, an IgE-mediated mechanism does not play a role.

Asthma is a disease characterized by reversible obstruction of the airways or bronchi. This is often accompanied by nonspecific bronchial hyperresponsiveness, which is the tendency of the bronchi in asthmatics to constrict in response to a wide range of chemical, pharmacological or physical stimuli. A second important feature of the histopathology of asthma is the intense infiltration of the bronchial mucosa with inflammatory cells such as eosinophils, neutrophils, macrophages and lymphocytes. The major clinical symptoms of asthma are the episodic occurrences of coughing, dyspnea, wheezing and chest tightness, alone or in combination. The symptoms may vary from mild and almost undetectable to severe and even life threatening. Asthma affects 5-10% of the population. A recent study in The Netherlands revealed that among children as much as 20% were affected. The symptoms that have been found in asthmatics are characterized by early and/or late asthmatic reactions. The IgE-mediated early asthmatic reaction which is maximal at 15-30 minutes after provocation and resolves within 1-2 hr is induced by allergen inhalation. This reaction is due to the release by mast cells of bronchoactive substances such as histamine and leukotrienes. The late asthmatic reaction which begins at 3-4 hr, is maximal at 6-12 hr and generally resolves itself within 24 hr, is correlated with cellular inflammation in the airways.

As mentioned above, atopic asthma is characterized by an increase in allergen-specific IgE antibodies. The mechanism of atopic asthma resembles a classical Type I hypersensitivity reaction (definition by Cooms and Gell). This reaction is schematically depicted in *figure 1*. In short, upon first contact with the allergen (sensitization), the allergen is taken up by antigen presenting cells (APC) and presented through major histocompatibility complex (MHC) class II molecules to T lymphocytes. These T lymphocytes produce cytokines which are required for B cell proliferation and differentiation which in turn lead to the production of allergen specific IgE antibodies. The IgE antibodies bind, via the FceRI receptor, to mast cells, thus sensitizing them. Further

Mast cells in asthma

Mast cells are not found in the circulation, they are resident in body tissues. Two types of mast cells, mucosal and connective tissue mast cells, have been identified through their different staining properties and biochemical characteristics (313). In humans, the connective tissue mast cell is found in serosal tissue and in skin, while the mucosal type predominates in the lung and in the mucosa of the gut. They are often localized in close contact to blood vessels, nerves, epithelial cells and fibroblasts, which are all potential targets for mast cell derived mediators. Mast cells contain granules in which the preformed mediators are stored, including biogenic amines (histamine, serotonin), proteoglycans (heparin), neutral proteases and acid hydrolases (290). In addition, mast cell mediators can be generated upon cell activation. Some of the *de novo* formed mediators are leukotrienes, prostaglandins D_2, platelet-activating factor, tumor necrosis factor–α (TNF–α), transforming growth factor–β (TGF–β), chemokines, interleukin-3 (IL-3), IL-4 and IL-5. During allergic reactions mast cells are activated by cross linking of IgE molecules bound to the FceRI receptor. Other important immunological triggers of mast cell secretion include IL-1, IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF) and a family of histamine-releasing factors (313). Additionally, numerous nonimmunological stimuli are also important in the activation of human mast cells, such as complement fragments C3a, C4a and C5a, various neuropeptides neurotransmitters (adenosine triphosphate) and various drugs such as opiates (313).

An important role for mast cells in allergic asthma has already been established. Mast cells and mast cell products in the bronchoalveolar lavage (BAL) fluid have been shown to be important in the early phase responses in allergic asthma (270, 316). Moreover, bronchial biopsy studies in patients with allergic asthma revealed increased numbers of mast cells, which diminished following allergen provocation due to the loss of their metachromatic granules (157, 237). From these studies it is evident that mast cells play a role in the immediate allergic reaction. In addition, recent data have suggested an important role for mast cells in the development of late asthmatic reactions. It has been demonstrated that bronchial hyperreactivity was correlated with mast cell mediators in the BAL fluid in allergic patients (54, 55). Moreover, it appeared that mast cells are capable of releasing proinflammatory cytokines, such as IL-4 and TNF–α (36, 225). These and other proinflammatory cytokines are capable of attracting inflammatory cells such as eosinophils, neutrophils and T lymphocytes into the tissue (103).

also capable of releasing cytokines. It has been demonstrated that mast cells are involved in the development of allergic inflammatory responses through the release of IL-4 and TNF-α (36, 225). Additionally, airway epithelial cells, fibroblasts (68, 99), alveolar macrophages (109, 113) and eosinophils (41) have been described to release cytokines during asthmatic events.

Eosinophils and neutrophils in asthma

The influx of inflammatory cells in the airways is associated with the late asthmatic reaction. Eosinophils were found in high concentration in sputum, BAL fluid and tissue biopsy specimens in human asthmatics (26). Moreover, eosinophil numbers were further enhanced after local allergen provocation (148, 179). It is generally accepted that the eosinophil is a major player in allergic asthma. However, the contribution of the other inflammatory granulocyte cell type, the neutrophil, should not be overlooked. Its role in allergic asthma however, is still poorly understood (284). The neutrophil has a tremendous potential to generate inflammation by its release of lysosomal enzymes, oxygen metabolites, leukotriene B_4 and the generation of histamine-releasing factor (317). Indeed, after allergen provocation, neutrophils have been detected in increased numbers in the BAL fluid during the late phase bronchial response (71, 202). Fabbri and coworkers compared leukocyte counts in sputum, bronchial biopsies and BAL fluid and found that in bronchial asthma the percentages of neutrophils were significantly higher in sputum than in BAL (170). This observation could explain the lack of research on the role of the neutrophil in asthma since BAL and biopsy studies are more customarily used than sputum analysis. Interestingly, Anticevich and coworkers recently demonstrated that neutrophils were capable of inducing *in vitro* airway hyperresponsiveness in sensitized human isolated bronchial tissue (6). Furthermore, it has been demonstrated that the late asthmatic reaction in guinea pigs was dependent on the presence of neutrophils (214).

The inflammatory response in the late phase reaction of allergic asthma is thought to be relevant for the development of airway hyperresponsiveness. Airway hyperresponsiveness is an exaggerated constrictor response to various stimuli, and is considered a key feature of asthma which correlates with the severity of disease, frequency of symptoms, and need of treatment (260). The relationship between airway hyperresponsiveness and airway inflammation in asthma has been studied extensively but remains unclear. Several human studies suggested a binding association between the two phenomena (1, 26, 153). However, it has also become clear that bronchial hyperresponsiveness is not dependent on airway inflammation and vice versa (243, 260). The nondependency of these two

studies (mostly in animal models) have also demonstrated the ability of sensory neuropeptides to mediate inflammatory responses and bronchial hyperresponsiveness. For example, *in vitro* studies of vascular endothelial cells demonstrated that SP induced intercellular adhesion molecule-1 (ICAM-1) expression which in turn enhanced neutrophil migration (216). This proinflammatory effect of SP was also established using bronchial epithelial cells. SP stimulation of neutrophils induced a dose-dependent adherence of neutrophils to bronchial epithelial cells (70). Additionally, in human dermis SP induced a rapid influx of neutrophils and eosinophils which was associated with an upregulation of adhesion molecules (283).

In the airways, administration of SP or NKA by inhalation or intravenous administration has been reported to cause a bronchoconstriction (reviewed in 130). In these studies, NKA was found to be a more potent bronchoconstrictor than SP, and asthmatics were found to be hyperresponsive to SP and NKA. In addition, in conscious guinea pigs inhalation of SP or NKA elicited respiratory effects, which could be inhibited by specific NK_1 and NK_2 receptor antagonists (152). In contrast to most mammalian species, in the mouse stimulation of the NK_1 receptor by SP and NKA in bronchial smooth muscle preparations results in a bronchodilation (174). Moreover, no NK_2 or NK_3 receptor activity has been detected in this species (174).

A valuable tool in neuropeptide research has been the neurotoxin capsaicin. Capsaicin induces an initial excitation resulting in massive release of mediators from unmyelinated sensory C-fibres (171). Inhalation of capsaicin resulted in a direct influx of eosinophils, neutrophils and mononuclear cells in the human nasal lavage fluid (240). Repeated systemic application of capsaicin in animal models leads to the depletion of all sensory neuropeptides (126). This procedure can be used to examine the role of sensory neuropeptides in various disease models. Systemic capsaicin pretreatment inhibited the development of bronchial hyperresponsiveness in several guinea pig models for allergic asthma (134, 149, 155, 230). In addition, it was described recently that capsaicin was a potent inhibitor of nuclear transcription factor-κB, which is a transcription factor important in the regulation of various pathological conditions including inflammatory reactions (280).

Animal models for atopic asthma

Several animal models have been utilized to investigate the pathogenesis of allergic asthma. The ovalbumin sensitized and challenged guinea pig is a frequently described model for bronchial hyperresponsiveness and eosinophilic infiltration (156, 192, 246).

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Ovalbumin inhalation caused an early and a late asthmatic reaction in conscious, unrestrained guinea pigs (271). The association of the eosinophil with the development of bronchial hyperresponsiveness (195, 246) and the late asthmatic reaction (257) has been determined. Furthermore, ovalbumin sensitization has been used in the mouse to induce allergic responses. Several groups including our own found that sensitization with ovalbumin (either by inhalation, topical application or intraperitoneal injection), followed by an aerosolized inhalation challenge resulted in the production of ovalbumin-specific IgE antibodies, bronchial hyperresponsiveness and eosinophilic infiltration in the airways (116, 255, 269). The role of Th2 cytokines in the development of these responses has been under extensive investigation. Coyle and coworkers demonstrated that ovalbumin immunization and allergen provocation led to lung T cells switching to a Th2 phenotype (65). Moreover, studies performed with neutralizing antibodies to IL-4 and IL-5 suggested a sequential involvement of these Th2 cytokines in the recruitment of eosinophils to the lung. IL-4 was secreted during immunization, switching naive $CD4^+$ T cells to a Th2 phenotype, which upon aerosol challenge were activated to secrete IL-5 leading to eosinophil accumulation (65). In another study Coyle and coworkers were able to inhibit the increases in IL-4 and IL-5 and the subsequent lung eosinophilia with an non-anaphylactogenic anti-IgE antibody administered 6 hr before antigen challenge (67). These results indicated that Th2 cytokine production and eosinophil infiltration in the mouse airways are IgE-dependent and suggested that neutralization of IgE antibodies might be a novel therapeutic approach to the treatment in allergic airway diseases. The Th2 cytokine dependency of allergic reactions was further ascertained by investigating the inhibitory activity of Th1 cytokines. In ovalbumin sensitized mice administration by aerosol of IFN–γ, a Th1 cytokine, 3 days prior to ovalbumin challenge inhibited the development of airway hyperreactivity (154). Furthermore, aerosolized recombinant IFN–γ prevented the ovalbumin-induced eosinophil recruitment into the airway by inhibiting $CD4^+$ T cell infiltration (217). Additionally, mice lacking the IFN–γ receptor (IFN–γR deficient mice) proved to be a suitable tool to investigate Th2 responses in the airway. It was found that in wild-type mice ovalbumin-induced eosinophilia in the airways was cleared 2 months after the challenge which was still present in IFN–γR deficient mice (66). These results suggested that endogenous IFN–γ had no effect on the development of lung eosinophilia, but was able to modulate the Th2 response (66).

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2 Delayed-type hypersensitivity reactions

The second group of asthmatics comprise the non-atopic, intrinsic asthmatics. In these patients no increase in allergen-specific or total IgE has been found. It has been hypothesized that in some of the intrinsic asthmatics this IgE-independent mechanism resembles a Type IV hypersensitivity or delayed-type hypersensitivity (DTH) reaction. Type IV hypersensitivity is used as a general category to describe all those hypersensitivity reactions which take more than 12 hr to develop and which involve cell-mediated immune reactions rather than humoral responses. In general, three types of DTH reactions are recognized. The classical DTH was first described by Koch who observed that subcutaneous injection with tuberculin resulted in the development of fever and generalized sickness associated with hardening and swelling of the skin at the site of reaction. The tuberculin hypersensitivity reaction starts with T cells ($CD4^+$:$CD8^+$, ratio 2:1) migrating out of the capillaries at 12 hr, followed by migration of macrophages and Langerhans' cells out of the epidermis at 48 hr. The cellular traffic continues over the next 24 hr, and class II molecules appear on keratinocytes; there is no oedema of the epidermis. Subsequently, tuberculin lesions may develop into the second type of DTH reaction, the granulomatous hypersensitivity. Granulomatous hypersensitivity is clinically the most important form of DTH giving rise to hardening of the skin or lung. Granuloma formation occurs where there is continuous stimulation due to persistent or recurrent infection, or where macrophages cannot destroy an antigen. The reaction time is maximal at 21-28 days. The third subtype of DTH is contact hypersensitivity. This subtype is similar to the tuberculin subtype in that it peaks 1 to 2 days after local antigen challenge (local second contact). In contrast, contact hypersensitivity is predominantly an epidermal reaction caused by contact allergens, such as nickel chromate and poison ivy. Contact hypersensitivity is characterized clinically by an eczematous reaction at the site of contact with the allergen. The cellular cascade of this subtype is discussed in more detail below.

Cellular reactions in contact hypersensitivity reactions

Although the basis of all subtypes of DTH reactions is the same this paragraph concentrates on the cellular reactions that occur in contact hypersensitivity (CHS) reactions. The reactions, schematically depicted in *figure 2*, are ascertained in murine studies. CHS is elicited by haptens, which are molecules that are too small to be antigenic themselves. These haptens penetrate the epidermis where they bind, mostly covalently, to normal body proteins. Two separate phases in CHS can be identified in which two

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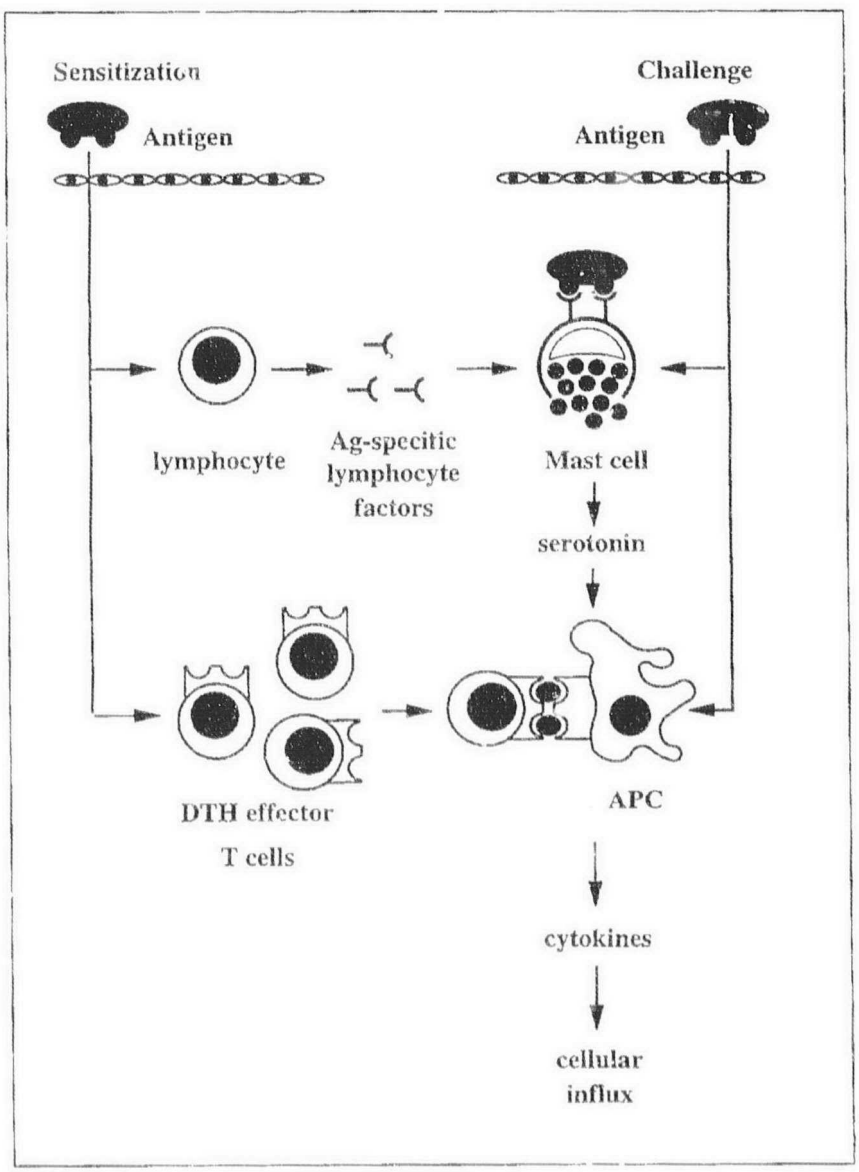

Figure 2 Schematic presentation of the cellular reactions in contact hypersensitivity (Type IV) reactions.

different types of lymphocytes are involved. During the first sensitization (or elicitation) phase specific DTH-initiating lymphocytes are generated within 1 to 2 days after the first contact with the hapten. These DTH-initiating lymphocytes are antigen-specific lymphoid precursor cells that arise before final differentiation along the pathway leading to mature T or B cells (115). They are stimulated to produce hapten-specific lymphocyte factors which circulate the body and infiltrate tissues, such as skin, lung and intestine. Subsequently, these lymphocyte factors
bind to mast cells and possibly other cells (150 . Indeed, Redegeld and coworkers recently demonstrated *in vitro* that the lymphocyte factor specific for picryl chloride, a low molecular weight sensitizing agent, was able to bind to mast cells and to a lesser extent to macrophages, fibroblasts and sheep red blood cells (252). Moreover, macrophages were activated by the picryl chloride-specific lymphocyte factor to produce nitric oxide (NO), which may be in line with the suggestion that NO could have a role in the elicitation of CHS (253). Upon second contact with the hapten (challenge or effector phase) the hapten binds to the lymphocyte factor bound to the mast cell. Through this association the mast cell is triggered to release its mediators (198). One of these mediators, serotonin, gives rise to an increased microvascular permeability and an increased expression of adhesion molecules (295). These events lead to the infiltration of DTH effector T cells which were generated during the sensitization phase (11). DTH effector T cells can recognize the antigen in the context of MHC class II on the APC which is followed by the production of cytokines. These cytokines attract other leukocytes to the site of antigen exposure resulting in a DTH response (11).

In summary, DTH reactions seem to consist of a sequential cascade of steps that include at least two different types of lymphocytes and also mast cells, endothelial cells, antigen presenting cells and bone-marrow derived circulating leukocytes (*figure* 2). Two components can be recognized: an early 2 hr response and a later 24 hr response after challenge, which are mediated by DTH-initiating lymphocytes and DTH effector T cells respectively.

T lymphocytes and cytokines in DTH

It is generally accepted that the T lymphocytes involved in DTH reaction belong to the $CD4^+$ Th1 subset. *In vitro* and *in vivo* antibody depletion studies have indicated the need for immune $CD4^+$ T cells for elicitation of CHS in the skin (101, 204). Furthermore, the generation of specific $CD4^+$ T cell clones from the cutaneous lesions of patients with allergic contact dermatitis to nickel and cobalt has supported the role of $CD4^+$ T cells in

CHS (161, 281). More recently, it has been demonstrated that in mutant mice lacking cell surface expression of the CD4 molecule ($CD4^-$) the CHS to the low molecular weight hapten dinitrofluorobenzene (DNFB) was significantly decreased 24 hr after the challenge. Moreover, the early skin swelling response 1-3 hr after the challenge was completely abolished in $CD4^-$ mice (146). Additionally, other models, such as oxazolone-induced CHS in the murine oral epithelium (78) and picryl chloride-induced DTH in murine liver (321), have suggested an important role for $CD4^+$ T cells in the induction of cell-mediated immunity.

Investigations of cytokine expression and production indicated that these $CD4^+$ T cells belonged to the Th1 subset. In biopsies from patients with allergic contact dermatitis increased expression of IFN–γ mRNA was detected (122). In contrast, in biopsies from patients with atopic dermatitis increased expression of IL-10 (a Th2 cytokine) has been observed (227). Th2 cytokines, such as IL-4 and IL-10, have been demonstrated to inhibit DTH reactions. For example, IL-10 inhibited the elicitation phase of DNFB-induced CHS (147, 277) and additionally the Th1-induced granulomatous hypersensitivity reaction in mice (159). Moreover, mice lymph node cells ($CD4^+$ and $CD8^+$) isolated 1 day after immunization with picryl chloride produced IFN–γ after stimulation with IL-2 which could be blocked by coculture with IL-4 (72). To further support an inhibitory role for Th2 type cytokines, IL-4 has been shown to inhibit the dinitrochlorobenzene (DNCB)-induced CHS reaction when administered at the time of challenge (effector phase) whereas treatment with anti-IL-4 antibodies markedly increased the magnitude of CHS (100). Additionally, anti-IL-4 antibody enhanced the expression of IFN-γ mRNA and subsequently the development of DTH responses whereas anti-IFN–γ antibodies partial inhibited the DTH responses in immunized mice given pertussis toxin (211).

Although the above data suggest that DTH-type reactions are Th1 dependent, other studies have suggested that this conclusion is not correct (212). For example, in skin biopsy specimens from atopic dermatitis patients (Type I hypersensitivity) and patients with a positive Mantoux test (tuberculin-induced DTH) an increased expression of Th1 cytokine mRNA and Th2 cytokine mRNA was detected, respectively (300). Furthermore, it has also been suggested that Th2 cytokines, especially IL-4, are important for the induction of CHS reactions. Two independent groups demonstrated the production of IL-4 mRNA in draining lymph nodes 3-4 days after sensitization (119, 205), indicating that IL-4 could be involved in CHS reactions. Involvement of both IFN–γ and IL-4 in the generation of oxazolone-specific T cell responses in mice has also been demonstrated (298). In human studies, nickel-induced contact dermatitis was associated with an increase in IL-5, another

Th2 cytokine, and variable amount of IFN–γ and Il -4 suggesting a Th2 or Th0 response (247). Furthermore, using monoclonal antibodies to IL-4, Salerna and coworkers showed an essential role for IL-4 in the effector phase of trinitrochlorobenzene (TNCB)-induced CHS (268).

In addition to the controversy over whether DTH reactions are Th1 or Th2 reactions, current studies have raised considerable doubt about the exclusive role of $CD4^+$ Th1 cells in the induction of DTH responses. A wealth of evidence has been gathered to support a significant role for $CD8^+$ T cells in the cellular cascade of DTH reactions. *In vivo* depletion of $CD8^+$ T cells prior to sensitization with DNFB and oxazolone resulted in the complete inhibition of a DTH response (107). Surprisingly, depletion of $CD4^+$ cells resulted in a strikingly enhanced ear swelling response. Expression of the chemokine IFN–γ inducible protein (IP-10) mRNA was increased during CHS responses (induced by DNFB and oxazolone) and was primarily mediated by $CD8^+$ T cells (2). To try and understand a role for $CD8^+$ T cells it is important to understand that there are functionally distinct subsets of $CD8^+$ T cells that produce different combinations of cytokines. Cells of the type 1 $CD8^+$ T cell subset (Tc1) secrete IFN–γ but not IL-4 and are restricted by MHC class I; type 2 $CD8^+$ T cells (Tc2) secrete IFN–γ, IL-4 and IL-10 but not IL-6 and are restricted to MHC class II (142). Based on these data it was postulated that cytotoxic/suppressor $CD8^+$ T cells can also perform Th1 and Th2 like activities (142).

Taken together, these results clearly demonstrated that both $CD4^+$ and $CD8^+$ T cells could play a role in DTH reactions but that the partial contribution of each needs to be elucidated. The conflicting data probably depends on the particular type of DTH response, species used, and experimental setup. It is however likely that, especially in humans, the balance between $CD4^+$/$CD8^+$ and Th1/Th2 or Tc1/Tc2 is extremely delicate.

The role of mast cells in DTH

The role of the mast cell in asthma has been investigated extensively. It has long been known that mast cell products (tryptase, histamine) can be identified in the blood, urine, BAL fluid and/or lung tissue of patients with asthma (314). An increased number of mast cells was detected in the airways of both atopic and non-atopic asthmatics (106). However, the precise contribution of mast cells in the development of asthmatic responses, i.e. airway hyperresponsiveness and inflammation, has not yet been elucidated.

Askenase and coworkers demonstrated that mast cells are involved in the early phase of a great percentage of DTH reactions in the skin elicited by picryl chloride, oxazolone, DNFB and nickel sulfate (125, 307). The picryl chloride-induced accumulation of

mononuclear cells around bronchioli and blood vessels in the lung was also severely reduced in mast cell deficient W/W^v mice and blocked by two serotonin (5-HT) receptor antagonists, methysergide and ketanserin (96). It was suggested that serotonin, which is stored and released from mast cells, activates endothelial cells resulting in an increased vascular permeability and enhanced expression of adhesion molecules allowing passage of DTH effector cells into the tissue (276). In addition, serotonin is capable of activating these DTH effector T cells via their functional 5-HT_2 receptor to produce cytokines (4). Additionally, it was demonstrated *in vivo* that mast cell degranulation was a key event in early evolving cutaneous DTH reaction in humans (144).

Mast cell deficient mouse strains (W/W^v and Sl/Sl^d) have been used to certify a role for mast cell in several DTH responses. The mast cell deficiency of W/W^v and Sl/Sl^d mice reflects distinct mutation of different chromosomes. The dominant white-spotting (W) locus on mouse chromosome 5 encodes the tyrosine kinase growth factor receptor, *c-kit*, whereas the steel (Sl) locus on chromosome 10 encodes the ligand for *c-kit*, also known as stem cell factor, mast cell growth factor or *kit* ligand (90). In contrast to suppressed DTH responses, normal and even higher than normal skin responses have also been obtained in the same two strains of mast cell deficient mice (91, 112, 199, 296). Geba and coworkers provided evidence that in mast cell deficient mice platelets can act as a source of serotonin instead of the mast cells because specific depletion of platelets markedly suppressed CHS responses to picryl chloride or oxazolone in both W/W^v and Sl/Sl^d mice (102). Furthermore, recent studies have demonstrated that repeated application of DNCB to the ears of W/W^v and Sl/Sl^d mice caused significant numbers of dermal mast cells to accumulate in the ears of W/W^v mice but not in Sl/Sl^d mice (143).

In addition to classical mediators, mast cell are able to act as a source of cytokines. It was demonstrated that mast cells in human bronchial biopsies contained IL-4, IL-5, IL-6 and TNF–α (38, 37, 224) and that isolated human lung mast cells had an increased expression of mRNA for IL-4 and IL-5 after IgE triggering (228). Furthermore, human mast cell lines produced another cytokine, IL-8 after nonspecific stimulation (206), and murine mast cell lines produced IL-3, IL-4, IL-5, IL-6 and GM-CSF after cross linking of FceRI receptor or after treatment with calcium ionophores (319). There have been no studies to date which have investigated the release and actions of mast cell derived cytokines in DTH reactions.

The role of sensory nerves in DTH reactions

In contrast to allergic reactions, the role of the sensory nerves in DTH reactions is poorly understood. In two models for DNFB-induced DTH reaction in the mouse lung and small intestine the sensory neuropeptides were involved in the elicitation of these reactions (47, 48, 151). Depletion of sensory neuropeptides resulted in total suppression of *in vitro* tracheal hyperreactivity whereas blockade of the NK_1 receptor resulted in the suppression of cellular accumulation in the airways and inhibition of vascular permeability in the airways and the small intestine. A CGRP antagonist ($CGRP_{8-37}$) had no effect on either responses (48). In contrast, mice depleted of sensory neuropeptides showed enhanced DTH skin swelling responses to DNFB suggesting that part of the effect of the sensory nerves was anti-inflammatory (48); which neuropeptides are important in this activity is unknown. In conclusion, there appears to be a fine balance between the pro- and anti-inflammatory action of sensory nerves and neuropeptides during DTH responses and the specific responses may be site dependent.

The two important cell types in the induction of DTH reactions are the mast cells and T lymphocytes (chapter 2.2 and 2.3). The interaction of both these cell types with sensory neuropeptides has also been described. It has been shown that SP and NKA have a predominantly stimulatory activity on T cell proliferation, migration and activation, whereas CGRP, VIP and SOM have a predominantly inhibitory activity (221, 311). In addition, both receptors for SP, the NK_1 receptor (250), and for VIP, the VIP-R_1 receptor (129) have been demonstrated on T lymphocytes. With regard to the mast cell, the close proximity of this cell type with sensory nerves in the skin, lung and intestine is also highly suggestive of an important interaction during an immune response. It has been demonstrated that SP can cause histamine release from human lung and skin mast cells (49, 62, 114) and serotonin release from murine lung and skin mast cells (104, 124). Interestingly, SP-induced mediator release from the mast cell is thought to be relayed via a different intracellular biochemical pathway than the pathway induced by anti-IgE antibodies (62). Moreover, NK_1 receptors have actually been located on mast cells and this receptor may be used in mast cell release function (131). In addition, other investigators published the possibility of SP to activate mast cells via a non-receptor mediated pathway by the direct interaction between cationic residues on the peptide and complementary charged structures on the mast cell surface (*figure 3*) (12).

Through their interactions with T lymphocytes and mast cells, sensory neuropeptides are also capable of influencing cytokine secretion patterns. It was demonstrated that SP can increase mRNA for TNF-α and secrete TNF-α in a mouse mast cell line (63)

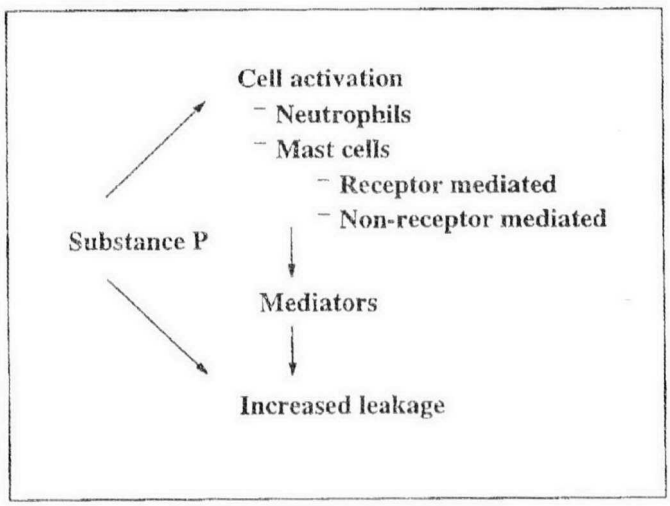

Figure 3 Biological activities of substance P

however, the expression of IL-3, IL-4, IL-6 and GM-CSF was not effected by SP. On the other hand, SCM can inhibit the release of TNF–α, IL-1b and IL-6 from human monocytes (235) and VIP can downregulate IL-2-, IL-10- and IL-4 mRNA in T cells (311). These data demonstrate that sensory neuropeptides can differentially regulate cytokine release during an immune response.

3 Occupational asthma

The definition of occupational asthma has been a point of discussion for many years between different groups. The one recommended by Bernstein and coworkers (28) is very plausible: *it is a disease characterized by variable airflow limitation and/or nonspecific bronchial hyperresponsiveness due to causes and conditions which are attributable to a particular occupational environment and not to stimuli encountered outside the workplace.* Occupational asthma has become the most prevalent occupational lung disease in the developed countries (reviewed in 59). The incidence of occupational asthma is largely dependent upon the causative agent. In some industries even more than 50% of the exposed workers develop occupational asthma; this particularly applies to platinum salts

and detergent enzymes (110, 302). In general, it is estimated that between 5 to 10% of all cases of adult asthma are occupational in origin (range from 2% of all cases in the United States to 15% of all adult cases in Japan) (23, 201). However, this estimation could still be flattering since occupational asthma is difficult to diagnose without the proper investigations (17, 53). Until now, over 200 causes of occupational asthma are recognized and more are identified each year (59). They can be divided into two groups: those that are complete antigens, the high molecular weight agents, and the low molecular weight agents. In *table 1* some examples of both groups are listed with the respective workers at risk (taken from 59).

Table 1. Common agents that cause occupational asthma and workers who are at risk.

Agent	Workers at risk
High molecular weight agents	
Cereals	Bakers, millers
Animal-derived antigen	Animal handlers
Enzymes	Detergent users, pharmaceutical workers,
Gums	Carpet makers, pharmaceutical workers
Latex	Health professionals
Seafood	Seafood processors
Low molecular weight agents	
Isocyanates	Spray painters, insulation installers, manufacturers of plastics, rubbers, foam
Wood dusts	Forest workers, carpenters, cabinetmakers
Amines	Shellac and lacquer handlers, solderers
Fluxes	Electronics workers
Chloramine-T	Janitors, cleaners
Dyes	Textile workers
Persulfate	Hairdressers
Formaldehyde	Hospital staff
Acrylate	Adhesive handlers
Drugs	Pharmaceutical workers, health professionals
Metals	Solderers, refiners

High molecular weight compounds such as proteins, polysaccharides, glycoproteins and peptides can induce an allergic response by producing specific IgE antibodies (7, 43, 75, 197, 245). There is no difference in the pathogenetical mechanism of asthma due to occupational high molecular weight allergens or other common inhalant allergens encountered in the environment such as house dust or pollens ( ). For instance, in the baking industry it has been demonstrated that inhalation of buckwheat flour caused an early IgE-mediated bronchoconstriction (232). Low levels of flour dust generated an increased risk of respiratory symptoms and airway hyperresponsiveness (31, 121). In addition, occupational asthma induced by latex is also characterized by an IgE-mediated hypersensitivity reaction (299). Furthermore, immediate, late and dual asthmatic reactions due to latex products have been reported (43, 304, 305).

Low molecular weight compounds (MW<1kD) are the most common agents causing occupational asthma (50, 58). In some cases the compound has been shown to act as a hapten and combines with a protein to form a hapten-protein conjugate. Specific IgE antibodies to the hapten-protein conjugate have been demonstrated with acid anhydrides such as phthalic anhydride and trimellitic anhydride (22, 309, 315). However, the majority of low molecular weight compounds induce asthma by unknown mechanisms, because specific IgE antibodies to the offending agents have not been found found in only a small percentage of the patients. Some examples are asthma due to wood dusts such as western red cedar, amines, colophony and other fluxes (*table 1*). The most frequently used low molecular weight agents causing occupational asthma are isocyanates.

Interestingly, occupational asthma due to western red cedar exposure and isocyanate exposure are similar in several aspects: the clinical history, the prevalence of affected subjects in the industry, the predominant occurrence of late asthmatic reactions on inhalation challenge tests, the persistence of asthma despite removal from exposure in a large proportion of sensitized subjects, and the lack of atopic subjects (58). Occupational asthma due to western red cedar is the most common form of occupational asthma in the Pacific Northwest and affects 4-14% of the exposed population (57). It has been shown to be caused by plicatic acid, a low molecular weight compound present uniquely in wood (57). All subjects with plicatic acid-induced asthma exhibit nonspecific bronchial hyperresponsiveness and a bronchial inflammation which is characterized by elevated numbers of eosinophils and T lymphocytes (mostly $CD4^+$ although in some cases also an increase of $CD8^+$ T cells was found) (85, 173). Furthermore, specific IgG or IgE antibodies were detected in only about 20% of the patients, suggesting that other immunologic mechanisms than Type I hypersensitivity are involved (57, 84). *In vitro* studies with

bronchial biopsy specimens, BAL mast cells and peripheral blood basophils from patients with western red cedar-induced asthma have further strengthened this hypothesis. Chan-Yeung and coworkers demonstrated that plicatic acid released histamine from bronchial mast cells of most patients with western red cedar-induced asthma which could not be attributed to plicatic acid-specific IgE antibodies (84). In addition, the plicatic acid sensitivity of human lung fragments was not transferable by serum of patient with western red cedar-induced asthma (84).

Occupational asthma induced by isocyanates

Isocyanates have a wide application in the industrial and domestic environment; the annual world-wide production is in the range of 3 million tons (20). The isocyanate group (-NCO) can react with any compound containing "active" hydrogen atoms. This property makes diisocyanates extremely useful for the production of polyurethane foam, paint, coating material and adhesive manufacture. Due to the wide range of applications in many workplaces isocyanates have become one of the main causes of occupational asthma in the developed countries (20). The most commonly used diisocyanate is toluene diisocyanate (TDI, a mixture of 2,4 and 2,6 isomers (80:20)) and it was developed during the second World War (185). TDI is a liquid at room temperature, almost colourless and very volatile. Due to its volatility it has been replaced by the less volatile methylene diphenyl diisocyanate (MDI) for producing rigid polyurethane foams (215). Other diisocyanates such as hexamethylene diisocyanate (HDI, which is more volatile than TDI), naphthylene diisocyanate (NDI), isophorone diisocyanate (IPDI) and hydrogenated MDI (HMDI) also have commercial use (*figure 4*). In spite of the harmful health aspects of diisocyanates, they are all still widely used in most developed countries, since no other chemicals have been found with the same chemical properties but less damaging effects on human health. The current Occupational Safety and Health Administration (OSHA) standards for exposure to TDI are 0.15 mg/m^3 short term exposure limit (STEL) and 0.04 mg/m^3 time weighted average (TWA) which were set to protect against respiratory irritation and asthma (275). In spite of these safety precautions approximately 5 to 10% of workers exposed to TDI are affected according to several studies (27, 73, 118, 215, 238).

Although TDI-induced asthma is one of the most studied cases of occupational asthma, little is known about the mechanism underlying this disease. Immunological, nonimmunological, irritant and combinations of these mechanisms have been proposed for the development of TDI-induced occupational asthma. The irritant effect of TDI is probably due to exposures to high concentration caused by spills which causes irritation of

B 12

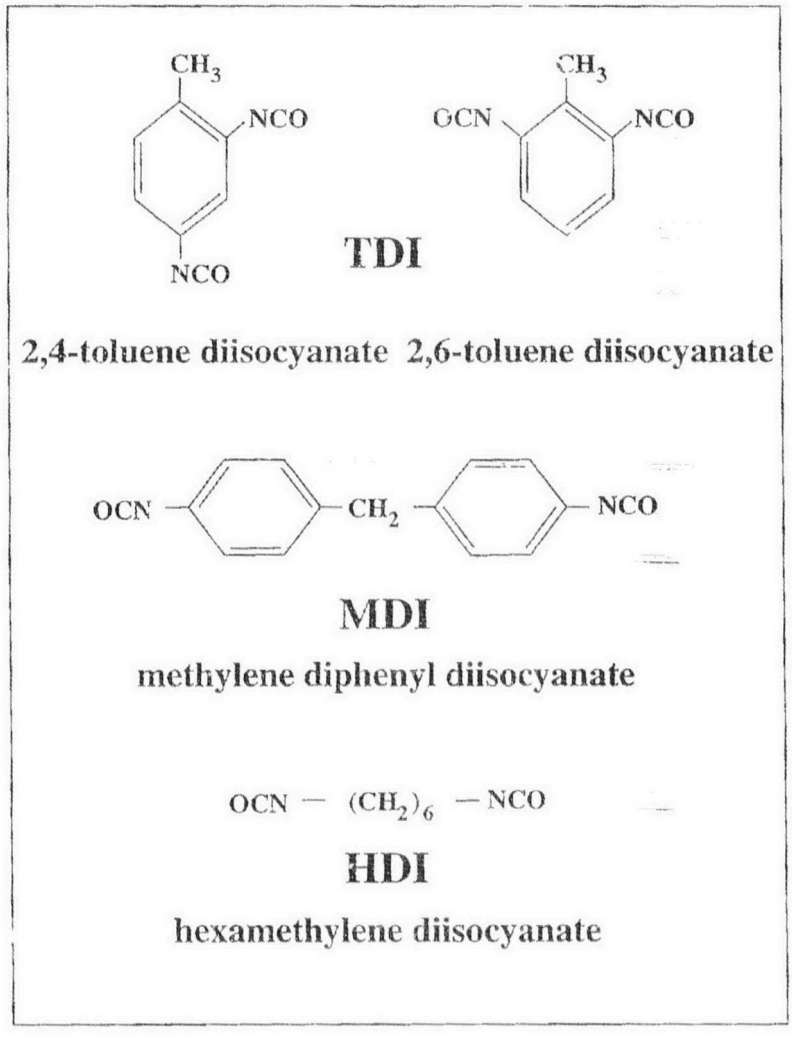

Figure 4 Chemical structures of isocyanates.

the eyes, nose, and pharynx in virtually all persons (286). The immunological mechanism of action of TDI was suggested by a number of clinical features: a) a latent period of exposure before the onset of respiratory symptoms, b) a minority of the exposed subjects developing sensitivity, c) once sensitized, workers react to inhalation concentrations at which control subjects show no respiratory reactions (323).

Subjects with TDI-induced occupational asthma can be divided into two groups: subjects with an increase in TDI-specific IgE antibodies in their serum (20% of the patients) and subjects with no increase in IgE antibodies in their serum (80% of the patients) (21, 135, 141). Based on these facts it is likely that TDI is capable of inducing asthma via different mechanisms (51). The IgE-mediated reaction resembles the mechanism of allergic asthma (paragraph 1) whereas for the IgE-independent reaction a cell mediated DTH-like reaction has been proposed (185, 186, 215). All subjects with TDI-induced occupational asthma exhibit airway hyperresponsiveness (238, 239) and dual and late asthmatic reactions occur more frequently than isolated immediate asthmatic reactions (16). The third important feature of TDI-induced asthma is inflammation of the airways. An increase in neutrophils and eosinophils in the BAL fluid of subjects with a late asthmatic reaction has been reported but not in subjects with an early asthmatic reaction (80, 230). Bronchial biopsy studies have also demonstrated a cellular influx in the airways of subjects with TDI-induced occupational asthma (24, 262, 288). Immunohistochemical analysis of bronchial biopsy specimens from subjects with TDI-induced asthma revealed an increase in $CD25^+$ cells (interleukin-2 receptor-bearing cells, presumed "activated" T-lymphocytes) as well as in total and activated eosinophils (24). Eosinophilic activation has also been demonstrated by increased levels of eosinophil cationic protein (ECP) in the serum of subjects with a late asthmatic reaction after exposure to TDI (184). In another study, it was demonstrated that TDI-induced asthma was associated with an increased number of total leukocytes and eosinophils in the airway mucosa (288). Interestingly, in this study the authors were also able to demonstrate an increased number of mast cells in the airway mucosa which was much greater in subjects who developed asthma after short-term exposure (2.4 ± 0.4 years) when compared with subjects who developed asthma after a much longer duration of exposure (21.6 ± 3.1 years) (288). This may reflect either a higher individual predisposition to the development of the disease or an increased response to TDI. Mast cell activation induced by TDI was further established by Sastre and coworkers who found an increase in serum neutrophil chemotactic activity (NCA; stored in mast cells) which is associated with mast cell or basophil activation, after bronchoprovocation-inhalation challenge with subirritant levels of TDI (272). Furthermore, proinflammatory cytokines such as TNF–α and IL-1b were increased in the submucosa of subjects with TDI-induced asthma as determined by immunohistology (169). Interestingly, the majority of T cell clones, derived from the bronchial mucosa of two patients with TDI-induced asthma, were $CD8^+$ producing IFN–γ and IL-5 in response to nonspecific stimulation (69). Moreover, the late asthmatic reaction was associated with an increase in

circulating $CD8^+$ T lymphocytes (83, 163). Based on these observations, it was speculated that an IgE-independent mechanism was involved, because atopic asthma is a $CD4^+$ T lymphocyte mediated reaction (5, 318). The TDI-induced late asthmatic reaction and the associated airway inflammation could be inhibited by prednisone (34, 81, 177). These data suggest an important role for airway inflammation in the induction of airway hyperresponsiveness (178).

Genetic factors might also play a role in the development of TDI-induced occupational asthma. It has been shown that human leukocyte antigen (HLA) complex products or genes may represent either a risk or a protective factor in the development of asthma (190). The first evidence that specific genetic factors might increase or decrease the risk of development of TDI-induced asthma was described by Bignon and coworkers (29). A more recent publication demonstrated that the gene HLA-DQB1*0503 has a role in conferring susceptibility to TDI-induced asthma (15). Inheritance of this genetic marker might therefore increase the risk of developing isocyanate-induced asthma, however, other factors, particularly environmental conditions, also play a crucial role.

Prevention of the development of TDI-induced occupational asthma is very important. It has been demonstrated that the end of occupational exposure to TDI is not always followed by a recovery from asthmatic symptoms (35). Cessation of exposure to TDI for 6 to 21 months resulted in reduced basement membrane thickening (264) and reduced number of subepithelial fibroblast, mast cells and lymphocytes in the bronchial mucosa (265). However, nonspecific bronchial hyperresponsiveness remained 30-48 months after the end of TDI exposure, suggesting permanent chronic damage to mechanisms controlling airway tone (231). Early diagnosis and early removal from exposure after the onset of asthma were demonstrated to be important factors for a favourable outcome of the disease (181, 242).

Animal models for TDI-induced occupational asthma

To study the mechanisms of action of TDI, animal models have been developed. Most of these models have been developed in guinea pigs and although they all used different sensitization/exposure regimes virtually all were associated with an increase in IgE or IgG antibodies (139, 138, 183, 213, 292). It has been demonstrated that TDI is capable of inducing airway changes by inhalation sensitization (61, 196, 251), topical sensitization (139, 292), intradermal sensitization (183) or intranasal sensitization (220) in the guinea pig. Moreover, airway changes induced by TDI inhalation have also been described in rats and rabbits (42, 188). Most of the described animal models for TDI-induced occupational asthma were associated with histological changes. In agreement with the TDI-induced

changes in human airways, an influx of eosinophils, neutrophils, mast cells and T lymphocytes has been demonstrated in these models (61, 183, 220, 251, 322). More recently, Karol and coworkers applied TDI topically to mice; they found an increase in TDI-specific IgG antibodies which was not associated with any histological changes, cytokine levels were unchanged but the effects on airway reactivity were not investigated (273).

In vitro experiments have proven to be very useful in investigating the effects of TDI. Mattoli and coworkers investigated the role of bronchial epithelial cell in the pathophysiological pathways of TDI-induced asthma. It was demonstrated that T. released the cytokines IL-1 and IL-6 (194) and 15-hydroxyeicosatetraenoic acid (15-HETE, a product of activation of the 15-lipoxygenase pathway) (193) upon incubation with bronchial epithelial cells, suggesting a significant contribution of these cells in TDI-induced occupational asthma. Based on other *in vitro* studies it was suggested that TDI caused occupational asthma through modulation of receptor sensitivity. Borm and coworkers showed that TDI enhanced the muscarinic response to methacholine in the trachea of the rat *in vitro* whereas the isoprenaline-induced relaxation was decreased (33). They hypothesized that TDI causes a dysbalance between bronchoconstrictive mechanisms (methacholine response) and bronchodilator mechanisms (β-adrenergic effects) (33).

Tachykinins are important mediators of inflammation in the airways of many mammals. Sheppard and coworkers were the first to demonstrate that TDI-induced airway hyperresponsiveness (2 hr after . hr exposure to 3 ppm TDI) was completely inhibited by treatment with capsaicin (to enable depletion of sensory neuropeptides) or the tachykinin antagonist (D-Arg^{1}, D-Pro^{2}, D-trp^{7,9}, Leu^{11}) substance P (297). They concluded that tachykinins were released into the intrathoracic airway during exposure to TDI (278). Furthermore, they also established an inhibitory effect of TDI on neutral endopeptidase (NEP) which lead to an increased responsiveness of guinea pigs to TDI (279). NEP is an important enzyme because it facilitates the breakdown of many peptides including SP. Mapp and coworkers found that TDI causes a concentration-dependent *in vitro* contraction of the rat isolated urinary bladder (179) and the guinea pig main bronchi (182). In both experimental setups the TDI-induced contractions were inhibited by capsaicin pretreatment (180) and a substance P antagonist and enhanced by a NEP inhibitor (182). In addition, the TDI-induced contraction of the guinea pig bronchi was inhibited by C48/80, a mast cell activating compound, but not by histamine H_1 and H_2 receptor antagonists (176) and increased levels of prostaglandins in the organ bath were established by radioimmunoassays (175). These results suggested that TDI activated the efferent function

of capsaicin-sensitive sensory nerves and that NEP may play a role in modulating the response in guinea pigs. More recently, Baur and coworkers also demonstrated that neuropeptides, especially the tachykinins SP and NKA, were important mediators of TDI-induced airway hyperresponsiveness in rabbits (189).

4 Aim and design of the present study

In the previous paragraphs it has become evident that the number of agents causing occupational asthma and subsequently the incidence of occupational asthma is rising. It has also become clear that the mechanisms of induction of occupational asthma are inconclusive. The aim of this present study is to develop a model (or models) for occupational asthma. More specifically, the mechanisms of action of TDI-induced occupational asthma are studied. The working hypothesis is that either DTH-like (Type IV hypersensitivity) reactions or IgE-dependent (Type I hypersensitivity) reactions are involved in TDI-induced occupational asthma in the mouse. Due to the extensive studies already completed in our laboratories concerning DTH-like pulmonary reactions using the low molecular weight haptens picryl chloride and DNFB, a mouse model for TDI-induced asthma was developed based on similar regimes. Chapter 2 describes the characteristics of this DTH-like model, mainly focusing on *in vitro* tracheal hyperreactivity and inflammatory responses.

In 70% of the subjects with TDI-induced asthma IgE antibodies are detected and appear to play a role in the development of the disease. In chapter 3, the TDI exposure regime is altered (long term exposure) is such a way that TDI-specific antibodies were produced. This IgE-mediated model for TDI-induced asthma is introduced and has characteristics similar to those found in atopic allergic models. The differences between the DTH-like (short term exposure) and atopic-like (long term exposure) models for TDI-induced occupational asthma are highlighted and discussed.

Historically many models investigating airway hyperresponsiveness have concentrated on IgE-dependent mechanisms and the literature on this subject far out ways literature on DTH-like mechanisms. Therefore, the following chapters of this thesis all focus on the DTH-like mechanisms involved in TDI-induced occupational asthma. First, in chapter 4 the role of the sensory nerves in the induction of TDI-induced airway changes is examined. Using capsaicin pretreatment and a specific NK_1 receptor antagonist the contribution of sensory neuropeptides during the sensitization and/or effector phase is analysed.

The effect of TDI-sensitization and challenge on mast cell function is outlined in chapter 5. Furthermore, the role of the mast cell in the induction of TDI-induced airway changes is investigated using mast cell deficient *W/W*^{v} mice. Lastly, the effect of ketanserin, a 5-HT_2 receptor antagonist, and cimetidine, a histamine H_2 receptor antagonist, are examined, to study the role of serotonin and histamine, respectively.

In chapter 6 and 7 the TDI-induced DTH-like reaction is further characterized. First, in chapter 6 the role of the T lymphocytes in the development of TDI-induced airway changes is presented using athymic nude mice. The specific subset of this T lymphocyte ($CD4^+$ or $CD8^+$) is identified using *in vivo* antibody depletion studies. Second, in chapter 7 the isolation and purification of a TDI-specific lymphocyte factor is described. Moreover, several parameters are measured to demonstrate the biological activity of this novel TDI-specific lymphocyte factor.

Finally, the conclusions of the different chapters are summarized and discussed in chapter 8.

C 04

CHAPTER 2

Toluene diisocyanate-induced *in vitro* tracheal hyperreactivity in the mouse

C 05

ABSTRACT

Toluene diisocyanate (TDI) is a low molecular weight compound which is known to cause occupational asthma in 5-10% of exposed workers. These patients exhibit marked airway hyperresponsiveness and granulocyte accumulation in the airways and 10-20% were also determined to have TDI-specific IgE in their serum. In this study we developed a murine model for TDI-induced asthma. After several sensitization and challenge regimes were tested, it was decided that optimal sensitization was observed after mice (BALB/c) were skin sensitized with TDI (1%) two times on two consecutive days and challenged intranasally 8 days later with TDI (1%). TDI-sensitized mice exhibited tracheal hyperreactivity to carbachol 24 hr after the challenge (69% increase in maximal contractile response). In contrast, no difference between the control and TDI-treated groups was observed 2 and 48 hr after challenge with 1% TDI. There appeared to be no elevation in TDI-specific IgE antibodies in the serum at all time points measured. In addition, no influx of leukocytes could be detected histologically in the trachea and lung tissue or airway lumen 2 and 24 hr after the challenge. Surprisingly, at 24 hr after the challenge, the tracheal hyperreactivity was associated with a marked increase in myeloperoxidase, but not eosinophil peroxidase, activity in the lung tissue and in the cells of the bronchoalveolar lavage fluid. To investigate the role of the lymphocytes in the induction of tracheal hyperreactivity mice were passively sensitized by intravenous injection of lymphoid cells from TDI-sensitized donor mice. Similar to active sensitization, adoptive transfer of lymphocytes from sensitized donors resulted in tracheal hyperreactivity 24 hr after challenge of the recipients.

In conclusion, these data show that TDI is capable of inducing lymphocyte-dependent but IgE-independent tracheal hyperreactivity in the mouse which is not associated with cellular infiltration in the airways. This model can be used to further investigate the possible mechanisms involved in the development of occupational asthma induced by TDI.

INTRODUCTION

Toluene diisocyanate (TDI) is a low molecular weight compound which is extensively used in industry, mainly in the production of polyurethane foams, automobile spray paints, varnishes and related products. Due to its wide distribution many people are exposed to TDI and over 100,000 workers are exposed in the United States alone (286). Virtually all

subjects exposed to high concentrations of TDI develop irritation of the eyes, nose and pharynx, but 5-10% of the workers also develop occupational asthma (27). However, for the development of occupational asthma it is not necessarily the concentration or duration of exposure per se but the product of both factors that is important for the progression of the disease (303). TDI-induced occupational asthma is characterized by specific airway hyperresponsiveness to TDI as well as an increase in nonspecific hyperresponsiveness. A second important feature of TDI-induced occupational asthma is inflammation of the airways. In patients neutrophil and eosinophil accumulation has been described (34, 178). The mechanisms underlying these symptoms are controversial. In only 20% of the subjects with TDI-induced asthma an increase in TDI-specific IgE antibodies has been reported (51). More recently, specific IgG antibodies have been shown in subjects with isocyanate-induced asthma (52). An IgE-mediated reaction is therefore not likely to be the only mechanism responsible for the action of TDI.

In the past, the guinea pig has been used extensively as a model to investigate TDI-induced asthma. The guinea pig model described by Karol and coworkers displayed both immediate and late onset airway responses (137). Since both responses may be consequences of antibody reactions, investigation of the mechanisms of TDI-induced asthma has focused on production and detection of TDI-specific antibodies. This model has demonstrated that antibody production was dependent on the concentration of TDI (136). In a more recent publication Karol and coworkers published a murine model for TDI-induced airway changes (273). This model focused on antibody production and an increase in IgG antibodies was observed after skin sensitization with TDI. In our laboratory several murine models have been developed to investigate IgE-independent reactions in the airways using other low molecular weight molecules, such as picryl chloride (94) and dinitrofluorobenzene (DNFB) (47). Skin sensitization with picryl chloride has been shown to induce tracheal hyperreactivity and pulmonary inflammation which were not associated with an elevation of serum IgE (94, 95). It is an interesting hypothesis that TDI-induced responses in the mouse and in man may also, under certain conditions, resemble this IgE-independent reaction. Both DNFB and picryl chloride have been extensively used to investigate delayed-type hypersensitivity (DTH) reactions in the skin and the lung. Upon sensitization with contact allergens (picryl chloride, DNFB) DTH-initiating T cells are activated to produce antigen-specific, antigen-binding T cell factors within 1 to 2 days (11, 307). The lymphocytes producing this factor can be isolated from draining lymph nodes and the spleen (307). The antigen-specific T cell factors can arm mast cells (and possibly some other cell types) (150, 198). Upon local second contact with the antigen (challenge)

the T cell factor armed mast cells are activated to release their mediators, especially vasoactive amines such as serotonin (11). Serotonin can induce infiltration of circulating antigen-specific DTH effector T cells ($CD4^+$ Th cells) (4), which are also generated during the sensitization (203). These DTH effector T cells can recognize antigen extravascularly in the context of MHC class II on antigen presenting cells after a second contact with the antigen. The DTH effector T cells are hereby triggered to produce cytokines leading to a DTH response (9)

In this study we developed a murine model to investigate TDI-induced occupational asthma. In the future, this model can be used to further clarify the mechanisms responsible for TDI-induced asthma.

MATERIALS AND METHODS

Animals

Mice (male BALB/c 6-8 weeks of age) were supplied either by the Central Animal Laboratory, Utrecht, The Netherlands or by the National Institute of Public Health and the Environment, Bilthoven, The Netherlands. They were housed in groups not exceeding 6 per cage and maintained under standard conditions. All experiments were assessed by the animal ethics committee at Utrecht University and the National Institute of Public Health and the Environment.

Sensitization procedures

Mice were sensitized once or twice daily on 1, 2, 3, 4 or 5 consecutive days either with 1% TDI (sensitized group) dissolved in acetone:olive oil (4:1) or with vehicle control (nonsensitized group) which was applied epicutaneously to the shaved abdomen and thorax (100 µl) and four paws (100 µl) (*table 1*). Intranasal administration was also used as a possible route for sensitization. Mice were sensitized intranasally with 20 µl 1% TDI dissolved in ethyl acetate:olive oil (1:4) or vehicle control twice daily on two consecutive days. In some experiments mice were sensitized with 100 µl 0.5% DNFB dissolved in acetone:olive oil (4:1) or vehicle control which was applied epicutaneously to the shaved abdomen and four paws on day 0 and day 1. During all sensitization procedures the mice were anaesthetized with sodium pentobarbitone (50 µl; 30 mg/kg i.p.). The optimal sensitization was determined to be skin sensitization with 1% TDI twice daily on day 0 and day 1.

Challenge procedure

Sensitized and nonsensitized groups were challenged with 1% TDI dissolved in ethyl acetate:olive oil (1:4) either on day 2, 6, 8 or 14 after sensitization (*table 1*). Twenty µl of the TDI solution was applied intranasally under light anaesthesia (sodium pentobarbitone; 50 µl; 30 mg/kg i.p.). After the optimal sensitization procedure had been chosen, different

concentrations of TDI for the challenge (0.01%, 0.1% and 1%) were tested. Furthermore, mice were also challenged on the ears; TDI (20 µl; 0.5%; dissolved in acetone) or vehicle control (20 µl) were applied topically to both sides of the ears. In some experiments to test for hapten specificity mice were challenged intranasally with 50 µl 0.6% dinitrobenzene sulphonic acid (DNS) dissolved in phosphate buffered saline (PBS, pH 7.2). The optimal intranasal challenge procedure was determined to be on day 8 with 1% TDI.

Isometric measurement of tracheal reactivity

Tracheal reactivity was measured using the method of Garssen and coworkers (97). Mice were killed by an overdose of sodium pentobarbitone (0.3 ml; 60 mg/kg i.p.). The trachea, which was resected *in toto*, was carefully cleaned of connective tissue using a binocular microscope. A 9 ring piece of the trachea (taken from just below the larynx) was then transferred to a 10 ml organ bath containing a modified oxygenated Krebs solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCL_2 6H_2O$, 0.5 mM $MgCl_2 6H_2O$, 25.0 mM $NaHCO_3$, 1.0 mM $NaHPO_4 H_2O$, 11.1 mM glucose). The trachea was directly slipped onto 2 supports of an organ bath, one of which was coupled to the organ bath and the other to an isometric transducer. The solution was aerated (95%: 5%; O_2: CO_2) at a constant temperature (37°C). Isometric measurements were made using a force displacement transducer (Harvard Bioscience, Boston, MA) and a 2 channel recorder (Servogar type SE-120) and were expressed as changes in mg force. Optimal pre-load for the mouse trachea was determined to be 1000 mg. The trachea was allowed to equilibrate for at least 1 hr before drug effects were elicited. During the equilibrium phase the fluid in the bath was changed every 15 minutes. To assess reactivity concentration-response curves to carbachol were determined 2, 24 and 48 hr after challenge with TDI.

Adoptive transfer of lymphoid cells

The inguinal lymph nodes and spleen from sensitized and nonsensitized mice were collected on day 6 after sensitization (twice daily on two consecutive days). They were pooled and cell suspensions were made by gently pressing the lymph nodes and spleen through a stainless steel screen. After washing the cell suspensions three times with cold PBS total cell counts were performed. Five x 10^6 or 10^7 lymphoid cells in 100 µl PBS were transferred into the retroorbital plexus of normal recipient mice under sodium pentobarbitone anaesthesia (50 µl; 30 mg/kg i.p.). Twenty four hr later these recipients were challenged intranasally with 1% TDI. Twenty four hours after this challenge tracheal reactivity to carbachol was measured.

Measurement of cutaneous reactions

An increase in ear thickness was measured 2, 24 and 48 hr after topical challenge with 0.5% TDI in acetone. Immediately after an i.p. overdose of sodium pentobarbitone the thickness of the TDI-treated ear and the vehicle-treated ear were measured using an engineers micrometer (Mitutoyo, Japan, No. 293-561) (48). Results are expressed as the difference in ear thickness (Δ ear thickness, mm) between the two ears.

Bronchoalveolar lavage (BAL) fluid

Lavage was performed in sensitized and nonsensitized mice 2, 24 and 48 hr after challenge. At the time of lavage the mice were killed with an overdose of sodium pentobarbitone (0.3 ml; 60 mg/kg i.p.). The chest cavity was exposed to allow for expansion after which the trachea was carefully intubated and the catheter was secured with ligatures. Phosphate buffered saline (PBS, 37°C) was slowly injected into the lung and withdrawn in 4 x 1 ml aliquots. The aliquots were pooled and then kept at 4°C. Total cells were counted using a haemocytometer and expressed as cells/lung. Leukocyte populations were then evaluated by staining BAL fluid lavage smears with a thiazine/eosin staining kit (Diff-quick). Results are expressed as leukocytes/lung for mononuclear cells, neutrophils and eosinophils.

Histological analysis

At 2, 24 and 48 hr after the intranasal challenge lungs and trachea were removed from mice after lethal anaesthesia with 50 µl of a cocktail consisting of 7 ml of 50 mg/ml ketalar, 3 ml 2% rompun, and 1 ml of 1 mg/ml atropine, injected intramuscularly (Nembutal causes vasodilatation, which negatively influences the histology preparations). Before removing the lungs the mice were perfused with 5 ml PBS (37°C) in the right heart ventricle. The lungs and trachea were filled intratracheally with a fixative (0.8% formalin, 4% acetic acid) using a ligature around the trachea. The unfolded lungs and trachea were fixed for at least 24 hr in the fixative, dehydrated, and embedded in paraplast. Four µm thick sections were stained with hematoxylin and eosin or with periodic acid Schiff reagent in combination with toluidine blue. An additional giemsa staining was performed to investigate the presence of eosinophils. Evaluation of the number of inflammatory cells was performed.

Enzymatic assays

Mice were killed with an overdose of sodium pentobarbitone and the lungs were perfused by injection of 5 ml PBS (37°C) into the right heart ventricle. Hereafter the lungs were weighed and homogenized in 2 ml cold tris-triton buffer (0.05 M tris-HCl, pH=8, 0.1% triton X-100). The homogenates were then exposed to freeze/thaw conditions 5 times whereafter they were centrifuged (1600 g, 5 minutes, 4°C). The supernatant was used for myeloperoxidase (MPO) and eosinophil peroxidase (EPO) measurements. After lung lavages the BAL fluid was spun (1600 g, 10 minutes, 4°C) and both the supernatant and the pellet were used for MPO and EPO measurements.

The MPO activity was assayed according to a standard spectrophotometrical technique (108). Serial dilutions were made in 96 wells microtiter plates, 50 µl sample was mixed with 150 µl phosphate buffer (0.05 M; pH 6.0) containing o-dianisidine dihydrochloride (0.167 mg/ml) and 0.0005% hydrogen peroxide. After 1 hr incubation at room temperature the absorbance at 460 nm was recorded. Results were expressed as optical density/g lung tissue or as optical density/g cells for lung tissue and BAL fluid samples, respectively.

The EPO activity was also assayed according to a standard spectrophotometrical technique (291). Serial dilutions were made in 96 wells microtiter plates, 50 µl sample was mixed with 100 µl tris-HCl (0.05 M, pH 8) containing o-phenylenediamine dihydrochloride (0.1 mM) and hydrogen peroxide (1 mM). After 1 hr incubation at room temperature the reaction was stopped by the addition of 50 µl 4 M sulphuric acid. The

absorbance was then determined at 492 nm. Results were expressed as optical density/g lung tissue or optical density/g cells for lung tissue and BAL fluid samples, respectively.

Chemicals

Toluene diisocyanate, olive oil, o-dianisidine dihydrochloride, o-phenylenediamine dihydrochloride were purchased from Sigma Chemical Co. St. Louis, U.S.A. Carbachol was purchased from Onderlinge Pharmaceutische Groothandel, Utrecht, The Netherlands. Dinitrobenzene sulphonic acid was purchased from Eastman Kodak, Rochester, New York. Sodium pentobarbitone was purchased from Sanofi, Maassluis, The Netherlands.

Statistics

All experiments were designed as completely randomized multifactorials with 6-14 mice per group. EC50-and Emax-values for the carbachol-induced tracheal contractions of each experimental animal were calculated separately by nonlinear least-squares regression analysis (simplex minimalization) of the measured contractions vs. carbachol concentration using the sigmoid concentration-response relationship and including a threshold value. The data were analysed by two-way Analysis of Variance followed by a post-hoc comparison between groups. In the figures and tables group means ± sem are given and a difference was considered significant when $P<0.05$. All data manipulation, non-linear fittings, Analyses of Variance and post-hoc comparisons were carried out with a commercially available statistical package (SYSTAT, version 5.03; Wilkinson L. SYSTAT: The system for statistics. Evanston, IL: SYSTAT, Inc., 1990. Statistics).

RESULTS

Tracheal hyperreactivity

Table 1 shows the Emax (maximal contractile response) values derived from concentration-response curves to carbachol (10^{-8}-10^{-4}M) 24 hr after the challenge following different sensitization procedures. The protocol where mice were sensitized twice daily on day 0 and day 1 and challenged on day 8, resulted in the most significant difference in tracheal reactivity to carbachol between the TDI-sensitized and nonsensitized groups. It is interesting to note that a marked increase in tracheal reactivity was also observed when the mice were challenged on day 2. After optimalization of the sensitization process, different challenge procedures were examined. Tracheal preparations taken from TDI-sensitized and nonsensitized mice were tested for hyperreactivity to carbachol (10^{-8}-10^{-4}M) 24 hr after intranasal challenge with either 0.01%, 0.1% or 1% TDI. The results in *figure 1* clearly show that the tracheal hyperreactivity 24 hr after the challenge was concentration dependent and both 0.1 % and 1% TDI gave a significant

Table 1. Different sensitization protocols

Skin sensitization 1% TDI	Challenge 1% TDI	Maximal tracheal reactivity to carbachol 24 hr after the challenge	
		Control	TDI
day 0 (1)	day 7	2290 ± 362	2273 ± 259
day 0, 1 (2)	day 6	2509 ± 404	1936 ± 69
day 0, 1; twice daily (4)	day 2	2138 ± 238	2967 ± 213*
day 0, 1; twice daily (4)	day 8	2094 ± 267	3041 ± 161**
day 0, 1, 2, 3, 4 (5)	day 14	2431 ± 196	2876 ± 213
day 0, 1, 2; twice daily (6)	day 7	2908 ± 256	2943 ± 498

Results are expressed as mean ± sem for n=3-10 mice/group.
( ): number of times of administration.
*: P<0.05 when compared with control.
**: P<0.01 when compared with control.

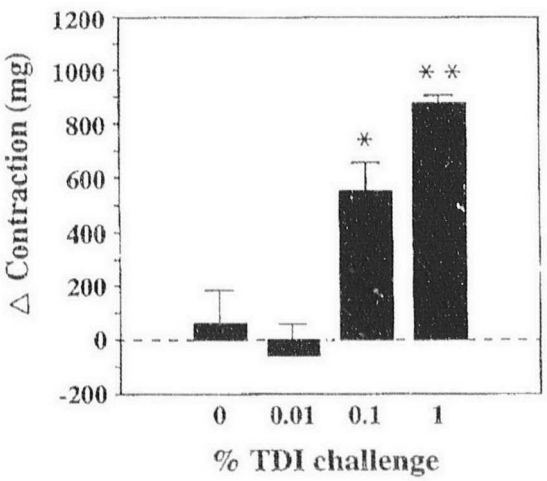

Figure 1 Difference (Δ) in maximal contraction (Emax sensitized mice - Emax nonsensitized mice) of trachea after completion of a concentration-response curve to carbachol. Responses were measured in trachea taken from TDI-sensitized and nonsensitized mice 24 hr after intranasal challenge with 0%, 0.01%, 0.1% and 1% TDI. Results are expressed as mean ± sem for n=6-10 mice/group. Significant differences between bars are denoted by (*) and (**) (P<0.05 and P<0.01 respectively).

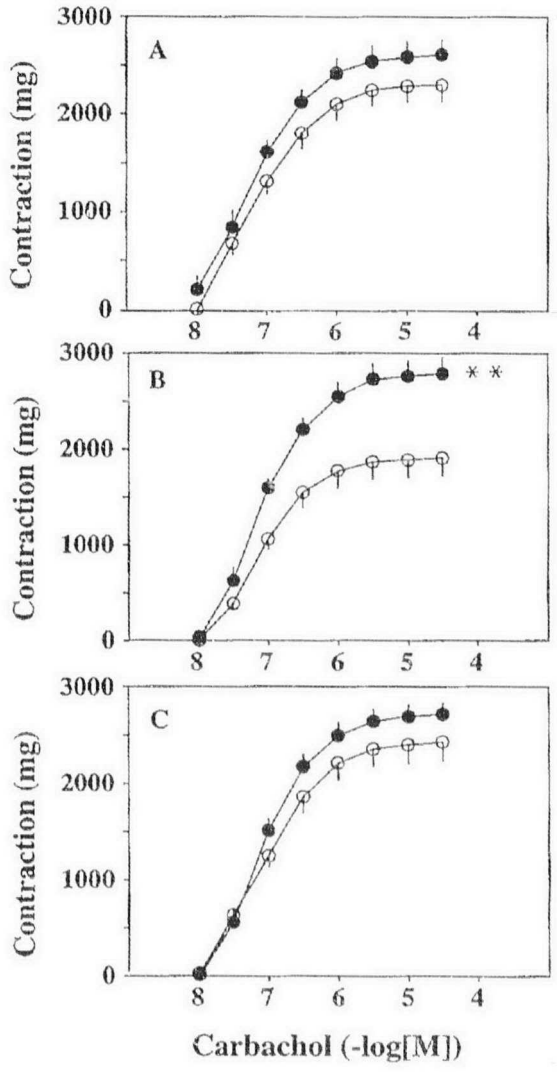

Figure 2 Tracheal reactivity A) 2 hr, B) 24 hr and C) 48 hr after the challenge. Concentration-response curves to carbachol were measured in the trachea of TDI-sensitized (closed circles) and nonsensitized (open circles) mice 2, 24 and 48 hr after intranasal challenge with 1% TDI. Results are expressed as mean ± sem for n=10-12 mice/group. Significant differences (P<0.01) between curves are denoted by (**).

($P<0.05$ and $P<0.01$, respectively) increase in Emax. In further experiments, tracheal reactivity at various time points after the challenge was examined using the optimal sensitization and challenge procedure. At 2 hr and 48 hr after the challenge with 1% TDI no significant difference was observed between the sensitized and nonsensitized groups (*figure 2A and C*). However, 24 hr after the challenge a marked and significant ($P<0.01$) increase in reactivity to carbachol was seen in the TDI-sensitized group compared with the nonsensitized group (*figure 2B*); in the TDI-sensitized group, when compared with the nonsensitized group, the Emax was enhanced by 69%. However, the EC50 (effective concentration that produced 50% of the response) for carbachol remained constant at all time points and all treatments (data not shown).

To test for hapten-specificity, TDI-sensitized mice were also challenged with a chemically unrelated hapten DNS. Twenty four hr after the challenge no tracheal hyperreactivity was observed in the TDI-sensitized-DNS-challenged group when compared with the DNFB-sensitized-DNS-challenged group (Emax: nonsensitized-TDI-challenged 1962 ± 307 mg; TDI-sensitized-TDI-challenged 2618 ± 365 mg; nonsensitized-DNS-challenged 1939 ± 236; DNFB-sensitized-DNS-challenged 2670 ± 320; TDI-sensitized-DNS-challenged 2127 ± 252 mg, mean ± sem for n=4-6 mice/group).

To examine which route of administration was most important for sensitization, mice were intranasally sensitized with 1% TDI or vehicle (twice daily, day 0 and day 1) followed by intranasal challenge also with 1% TDI on day 8. Using this protocol no tracheal hyperreactivity was observed in the TDI-sensitized mice when compared with the nonsensitized mice (*figure 3*).

In order to establish whether the induction of tracheal hyperreactivity in this model was dependent on the lymphocyte, we investigated whether this reaction could be transferred with lymphoid cells from TDI-sensitized donor mice. After pooling the cells from the inguinal lymph nodes and spleen, the same number of cells (5×10^6 or 10^7) from TDI-sensitized and nonsensitized mice were injected into naive recipient mice to study whether the tracheal hyperreactivity could be transferred. *Figure 4* shows that the mice that had received 5×10^6 lymphoid cells from TDI-sensitized donors exhibited tracheal hyperreactivity 24 hr after the challenge when compared with the control mice (Emax: nonsensitized donor group 2388 ± 166 mg; TDI-sensitized donor group 2822 ± 91 mg, mean ± sem for n=14 mice/group, $P<0.05$). When 10^7 lymphoid cells were transferred no significant difference in tracheal hyperreactivity between the sensitized and nonsensitized mice was found (Emax: nonsensitized 2348 ± 227 mg; TDI-sensitized 2658 ± 278 mg,

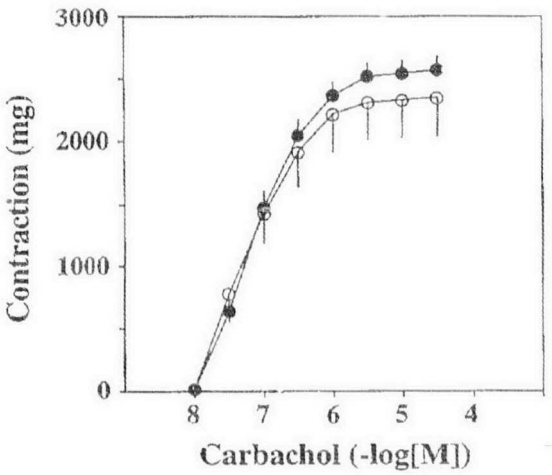

Figure 3 Tracheal reactivity 24 hr after the challenge with 1% TDI. Concentration-response curves to carbachol were measured in the trachea of intranasally TDI-sensitized (closed circles) and nonsensitized (open circles) mice 24 hr after intranasal challenge with 1% TDI. Results are expressed as mean ± sem for n=6 mice/group.

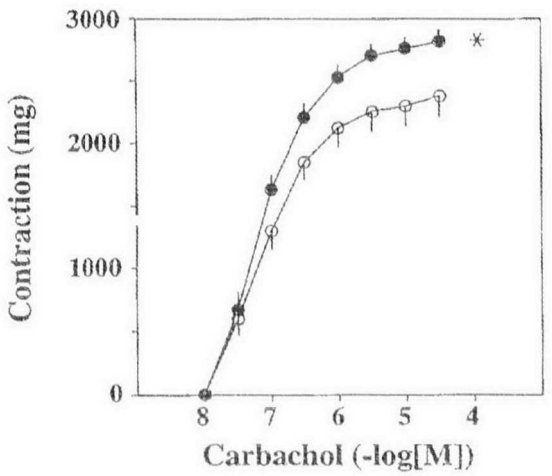

Figure 4 Adoptive transfer experiments. After adoptive transfer of lymphoid cells from TDI-sensitized and nonsensitized mice, donor mice were challenged intranasally with 1% TDI. Concentration-response curves to carbachol were measured in the trachea of passively sensitized (closed circles) and nonsensitized (open circles) mice 24 hr the challenge. Results are expressed as mean ± sem for n=14 mice/group. Significant differences (P<0.05) between groups are denoted by (*).

mean ± sem for n=6 mice/group). This was largely due to the fact that the control (nonsensitized) tracheal reactivity responses were increased.

Finally, TDI-specific IgE antibody in the sera was measured 2 and 24 hr after the challenge in nonsensitized and TDI-sensitized mice. Only one of the samples was measurable (TDI-sensitized, 24 hr after the challenge, n=5 mice/group); the other samples were below detection (personal communication Professor M.H. Karol, University of Pittsburgh, PA).

Cutaneous responses

In addition to measuring pulmonary responses induced by exposure to TDI, the development of cutaneous immune responses (i.e. contact hypersensitivity) was followed (*figure 5*). Two hr after the challenge on the ear there was no significant difference between the increase in ear thickness of both TDI-sensitized and nonsensitized groups. However, at 24 hr the increase in ear thickness of the sensitized group was significantly enhanced compared with the nonsensitized group ($P<0.01$). At 48 hr after the challenge there was still an increase of 50% in the sensitized group compared with the nonsensitized group, however this difference was no longer significant ($P=0.06$).

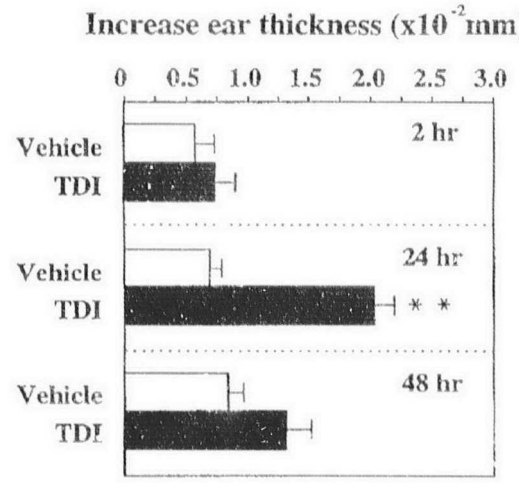

Figure 5 Ear swelling response 2, 24 and 48 hr after the challenge. TDI-sensitized (closed bars) and nonsensitized (open bars) mice were topically challenged on both ears; one ear with 0.5% TDI and the other with vehicle. The ear swelling was measured 2, 24 and 48 hr after the challenge using a micrometer and the difference in ear thickness (x 10^{-2} mm) between the two ears is expressed as mean ± sem for n=10-12 mice/group. Significant differences ($P<0.01$) between groups are denoted by (**).

Table 2. Total cell count in BAL fluid of sensitized and nonsensitized mice 2, 24 and 48 hr after challenge with 0.1% or 1% TDI.

Challenge	1% TDI (x10^5)	0.1% TDI (x10^5)
2 hr		
Control	2.51±0.30	3.86±0.81
TDI	2.97±0.31	2.67±0.59
24 hr		
Control	2.50±0.50	2.70±0.29
TDI	2.23±0.40	3.05±0.57
48 hr		
Control	2.43±0.22	3.41±0.76
TDI	3.35±0.19	3.40±0.35

Results are expressed as mean ± sem for n=10-12 mice/group

Pulmonary inflammation

In *table 2* the total cell numbers in the BAL fluid from TDI-sensitized and nonsensitized mice at 2, 24 and 48 hr after 0.1% or 1% TDI challenge are presented. No significant differences occurred at any of the time points between the TDI-sensitized and nonsensitized groups challenged with 0.1% or 1% TDI. Further, there were no significant differences between the leukocyte subtypes in the BAL fluid of TDI-sensitized and nonsensitized mice, challenged with 0.1% or 1% TDI, at all time points (*table 3*). In agreement with these data, no histological changes were found in the airway tissue taken from nonsensitized and TDI-sensitized mice. More specifically, no accumulation of inflammatory cells around bronchioli or pulmonary blood vessels was induced by exposure to TDI.

In further experiments, the MPO assay was used as a marker for neutrophils in lung tissue and in BAL fluid cells. In the BAL fluid the MPO assay was used as a marker for neutrophil activation. The MPO activity in the lung tissue of TDI-sensitized and nonsensitized animals at 2, 24 and 48 hr after 1% TDI challenge is shown in *figure 6A*. Two hr after the challenge there was no difference in MPO activity in the lung tissue between the sensitized and nonsensitized mice. However, 24 hr after the challenge the sensitized mice clearly showed an increase in MPO activity (368%) compared with the nonsensitized mice. This increase in MPO activity was largely resolved 48 hr after the challenge. Although the total amount of MPO present in the BAL fluid cells was much lower than in the lung tissue the results are in agreement with the MPO activity of

Table 3. Cell differentiation (%) in BAL of sensitized and nonsensitized mice 2, 24 and 48 hr after challenge with 0.1% or 1% TDI.

Challenge	1% TDI		0.1% TDI	
	Mononuclear	Neutrophils	Mononuclear	Neutrophils
2 hr				
Control	91 ± 3	8 ± 3	92 ± 3	8 ± 3
TDI	95 ± 4	5 ± 4	93 ± 2	7 ± 2
24 hr				
Control	95 ± 4	5 ± 4	95 ± 1	5 ± 1
TDI	95 ± 2	5 ± 2	93 ± 2	7 ± 2
48 hr				
Control	94 ± 2	6 ± 2	98 ± 1	2 ± 1
TDI	95 ± 3	5 ± 3	97 ± 1	3 ± 1

Results are expressed as mean ± sem for n=10-12 mice/group.

the lung tissue. *Figure 6B* demonstrates that there was no difference 2 hr after the challenge, however, a marked increase in MPO activity in the BAL fluid cells of the TDI-sensitized mice compared with the nonsensitized mice (673%) is observed 24 hr after the challenge. This effect had disappeared 48 hr after the challenge. In the BAL fluid there was no MPO activity detected at all time points measured and in all groups. In addition, the EPO activity was measured in the lung tissue, BAL fluid cells and BAL fluid 2, 24 and 48 hr after the challenge. In the lung tissue the EPO activity was detectable, however no difference was observed between the TDI-sensitized and nonsensitized groups (24 hr: nonsensitized-TDI-challenged 457.6 ± 64 Optical Density/g lung; TDI-sensitized-TDI-challenged 396.8 ± 64 Optical Density/g lung. 48 hr: nonsensitized-TDI-challenged 512 ± 96 Optical Density/g lung; TDI-sensitized-TDI-challenged 588.8 ± 64 Optical Density/g lung; mean ± sem for n=6 mice/group). In the BAL fluid cells and BAL fluid there was no EPO activity present in all groups tested.

DISCUSSION

In this present study, we have developed a murine model to investigate TDI-induced asthma. We used various sensitization and challenge procedures to find an optimal

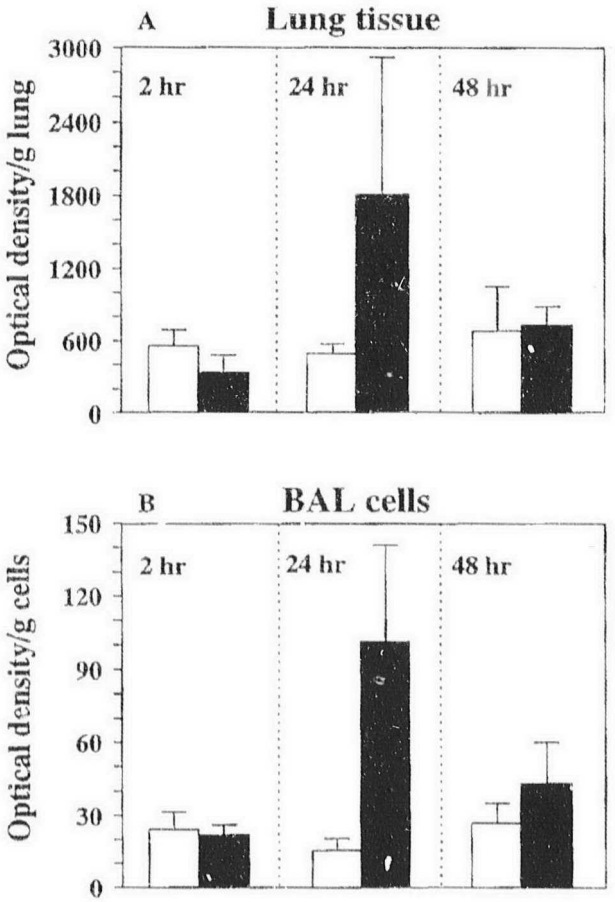

Figure 6 Myeloperoxidase (MPO) activity in A) lung tissue and B) BAL cells. MPO activity was measured in TDI-sensitized (closed bars) and nonsensitized (open bars) mice 2, 24 and 48 hr after the challenge using a standard spectrophotometric technique. The optical density/g lung or cells is expressed as mean ± sem for n=6-8 mice/group.

exposure to TDI. We demonstrated that skin sensitization with 1% TDI (day 0 and day 1, twice daily) and intranasal challenge with 1% TDI (day 8) resulted in the most significant tracheal hyperreactivity. In contrast, intranasal sensitization and challenge with 1% TDI did not result in any changes in tracheal reactivity. These results may reflect differences in diffusion or antigen presentation of TDI haptens in the lung and the skin.

Tracheal hyperreactivity was not accompanied by an increase in TDI-specific IgE antibodies. Recently, another murine model for TDI was published by Satoh and coworkers (273). In this model, sera were drawn 10 days after skin sensitization with 0.5% TDI followed by intranasal challenge. In agreement with our studies, they found no changes in TDI-specific IgE antibodies. Unfortunately, airway reactivity was not measured in their study. The fact that TDI-specific IgE antibodies could not be determined in both studies, is however, no clear evidence of its absence. It is possible that the level of TDI-specific IgE antibodies in the serum was too low to be detected because it was bound to mast cells or other cells. Conversely, several indications suggest that an IgE-mediated mechanism does not play a role in TDI-induced tracheal hyperreactivity. First, 10-12 days generally are required for IgE antibodies to be produced; in our model significant tracheal hyperreactivity was observed after challenge on day 2 - obviously too soon for IgE production. Second, an IgE-mediated mechanism usually is related to an early asthmatic response; with our model, we did not find any clear effect earlier than 24 hr after the challenge in the airways. In addition, no immediate response was found in the skin of TDI-sensitized mice. Third, an increase in IgE is usually accompanied by an influx of eosinophils into the airway tissue and lumen. In our model for TDI-induced asthma we were unable to detect eosinophils histologically in the tissue or in the airway lumen. Moreover, an increase in EPO activity, which is used as a marker for eosinophils (291), could not be detected in the tissue or in the BAL fluid. Taken together, these results suggest that an IgE-mediated reaction probably is not the mechanism involved in tracheal hyperreactivity after sensitization and challenge with TDI. IgE-independent mechanisms could therefore also be playing an integral part in the response to TDI in the mouse airways.

It is interesting to note that the cutaneous responses after topical challenge with TDI bear a marked resemblance to cutaneous DTH reactions induced by other low molecular weight haptens (47). No ear swelling was seen directly (<2 hr) after the challenge. However, the local ear swelling response was significantly increased 24 hr after the challenge in the TDI-sensitized mice when compared with the nonsensitized mice. Recent publications from our laboratory have shown that other low molecular weight chemicals, like picryl chloride and DNFB, are potent contact sensitizers but like TDI can also induce marked tracheal hyperreactivity in the mouse airways (47, 95). Garssen and coworkers (95) developed a model for IgE-independent asthma in murine lungs using the hapten picryl chloride. Picryl chloride proved to be capable of inducing tracheal hyperreactivity 24-48 hr after intranasal challenge. The T cell dependency of the tracheal hyperreactivity was

established by several methods, such as the use of nude mice, hapten specificity studies and adoptive T cell transfer experiments (94). In our study it was also demonstrated that tracheal hyperreactivity was observed in mice that had received lymphoid cells from TDI-sensitized donor mice. This is interesting because T cells have been shown to be activated in occupational asthma caused by isocyanates in the bronchial mucosa in humans (24). However, lymphoid cells collected from spleen and lymph nodes are a mixed cell population containing, in addition to lymphocytes, also mast cells, basophils, macrophages and B cells. Exactly which cell types ($CD4^+$, $CD8^+$, B cells) are important in the induction of tracheal hyperreactivity to TDI is not yet known but is currently under investigation using adoptive transfer studies and the selective depletion *in vivo* of T and B lymphocyte subsets. However, because sensitization on day 0 and day 1 followed by challenge on day 2 resulted in tracheal hyperreactivity 24 hr after the challenge, DTH-initiating T cells are possible candidates for the induction of tracheal hyperreactivity.

Another prominent feature of asthma is pulmonary inflammation. Furthermore, accumulation of inflammatory cells in patients suffering from TDI-induced asthma has been reported (34, 178); neutrophil and eosinophil accumulation was seen in the BAL fluid. Epithelial damage and thickening of the basement membrane was observed in mucosal biopsy specimens of the main or lobar bronchi, as well as an inflammatory reaction in the submucosa mainly represented by lymphocytes, eosinophils and neutrophils (230). After picryl chloride sensitization, a pronounced inflammatory response, which was characterized mainly by mononuclear cells but also some polymorphonuclear cells, was noted around the bronchioli and blood vessels 48 hr after the challenge (96). In our study, however, we were unable to observe an influx of leukocytes into the airway tissue or lumen. No increase in total number of leukocytes was measured in the BAL fluid, nor could an increased percentage in specific leukocyte subpopulations be detected in the airway lumen of TDI-sensitized mice. Moreover, histological examination of the lung tissue and trachea did not reveal any inflammatory loci which is in direct contrast to the results obtained after exposure to picryl chloride (94). In agreement, the murine model described by Satoh and coworkers (273) showed only minimal inflammatory infiltrates in the lungs of TDI-sensitized mice 48 hr after intranasal challenge. The infiltrates they did find consisted of mononuclear inflammatory cells around pulmonary blood vessels. Surprisingly, in our studies the MPO activity in the lung tissue and BAL cells was enhanced 24 hr after the challenge. In our department, other cells were tested for their MPO content. It was observed that all other cells (mast cells, macrophages, eosinophils and fibroblasts) had virtually no MPO content, confirming that MPO activity can be used as a

specific marker for neutrophils. However, no influx of neutrophils into the airways could be determined; therefore, the increase in MPO activity should be interpreted as an increase in MPO content per neutrophil. The significance of such an increase is not clear but may represent a different activation state of the neutrophils in mice that were sensitized and challenged with TDI. Alternatively, activated neutrophils could have been phagocytized by macrophages making them impossible to detect using our histological technique (158).

Conflicting data have been published regarding the relationship between pulmonary inflammation and the induction of airway hyperreactivity. Studies in asthmatic subjects sensitized with TDI indicate that bronchial hyperreactivity could be related to inflammation of the airways (34, 178). On the other hand, it has been demonstrated in several animal models that bronchial hyperreactivity is not dependent on bronchial inflammation (94, 241, 256). In this study the tracheal hyperreactivity was not correlated to an influx of leukocytes, but an increase in MPO activity in lung tissue and BAL cells did appear 24 hr after the challenge. It is therefore possible that neutrophil activation, and not infiltration, may be partially responsible for the induction of tracheal hyperreactivity.

The murine model that was developed in this study exhibits some of the features observed in TDI-induced asthma in humans. First, the tracheal hyperreactivity in this model appeared at 24 hr after challenge which is considered a late response. Second, the development of TDI-induced tracheal hyperreactivity is lymphocyte dependent. Third, the increased MPO activity suggests an important role for the neutrophil in this reaction, but this response needs to be examined in the future. Finally, the fact that TDI-specific IgE antibodies could not be consistently detected is also in agreement with the majority of patients suffering from TDI-induced asthma. There has been much discussion in recent years as to the nature of airway responses induced by TDI. An irritant pathogenesis, which has been described particularly in the skin, has been suggested and is not mediated through an immunological reaction (77). In our study we observed irritant responses directly after application of TDI in the skin and in the airways. However, in the present study a clear sensitization response, albeit IgE-independent, was observed in the airways after repeated exposure to TDI. Our data would therefore support the hypothesis that both types of responses are induced after exposure to TDI.

In conclusion, TDI is still used extensively in industry. The best way to prevent new cases of occupational asthma is, of course, to stop using isocyanates. However, this is not a likely event in the near future. Consequently, research needs to be focused on TDI-induced asthma and this model provides the opportunity to reveal the immunological background behind the problems caused by TDI and other isocyanates.

CHAPTER 3

Long term topical exposure to toluene diisocyanate leads to antibody production and *in vivo* airway hyperresponsiveness in mice

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Long term topical exposure to toluene diisocyanate leads to antibody production and *in vivo* airway hyperresponsiveness in mice

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ABSTRACT

Toluene diisocyanate (TDI) is a low molecular weight compound which is known to cause occupational asthma in 5-10% of exposed workers. Recently, we developed a murine model to investigate TDI-induced occupational asthma. Short term exposure to TDI (skin sensitization twice daily on day 0 and day 1 and intranasal challenge on day 8) led to a nonspecific tracheal hyperreactivity 24 hr after the challenge in TDI-sensitized mice when compared with nonsensitized mice, while no TDI-specific IgE antibodies were found in the serum. Since 20% of subjects with TDI-induced occupational asthma exhibit an increase in serum IgE antibodies we exposed mice for a longer period of time to investigate whether this procedure could induce TDI-specific antibody production in exposed mice. Long term exposure (skin sensitization on 6 consecutive weeks followed by intranasal challenge on week 7) resulted in the production of total IgE and IgG and TDI-specific IgE and IgG antibodies. Airway reactivity to various agonists was also measured *in vitro* and *in vivo* in long term exposed mice. TDI-sensitized mice exhibited *in vitro* tracheal hyperreactivity to carbachol 3 hr after the challenge when compared with the nonsensitized mice. Moreover, *in vivo* airway hyperresponsiveness to serotonin was found 3 hr after the challenge in TDI-sensitized mice. Interestingly, *in vivo* airway hyperresponsiveness was not observed at any time point in the mice exposed to TDI according to the short exposure protocol.

In conclusion, by altering the exposure time and or cumulative dosage of TDI different biological reactions can be elicited in exposed mice. This important finding might be a reflection of the diversity of symptoms found in patients suffering from TDI-induced asthma. Both the short exposure and the long exposure model will be useful to further investigate the mechanisms of action of TDI.

INTRODUCTION

Toluene diisocyanate (TDI) is a well known cause of occupational asthma. TDI-induced occupational asthma is characterized by specific airway hyperresponsiveness to TDI as well as an increase in nonspecific hyperresponsiveness (141, 231). A second important feature of TDI-induced occupational asthma is inflammation of the airways identified by an influx of lymphocytes, eosinophils and neutrophils (24, 178, 230). However, the pathogenesis of TDI-induced asthma is diverse. In general, in only 20% of the subjects with TDI-induced occupational asthma an increased level of serum TDI-specific IgE

antibodies can be detected (21). For the remaining 80% of the subjects an IgE-independent mechanism has been proposed. Most studies describing human cases of TDI-induced occupational asthma demonstrate that TDI is capable of inducing different types of immune reactions. The dose-dependency of exposure to TDI and the severity of asthmatic symptoms has already been established (123, 136). Interestingly, it was also found that the number of immediate reactions to TDI decreased progressively when the interval between the last occupational exposure and the specific challenge was increased (208). In addition, Mapp and coworkers described that after cessation of exposure to TDI, subjects who showed immediate or dual asthmatic reactions tended to recover as opposed to subjects who showed only late asthmatic reactions in whom no improvement was found (181).

Previously, we have described an IgE-independent, murine model for TDI-induced occupational asthma (274). Short term TDI exposure (skin sensitization twice daily on two consecutive days followed by intranasal challenge on day 8 with 1% TDI) did not lead to the production of IgE antibodies whereas a significant, nonspecific tracheal hyperreactivity was found 24 hr after the challenge in TDI-sensitized and challenged mice when compared with nonsensitized mice. Adoptive transfer studies in which lymphoid cells from TDI-sensitized mice were transferred into naive recipients suggested an important role for T lymphocytes in the induction of this tracheal hyperreactivity. The characteristics of our model for TDI-induced occupational asthma resembled several findings found in murine models for pulmonary delayed-type hypersensitivity (DTH) reactions developed earlier in our laboratories (47, 95). We therefore hypothesized that the induction of a DTH-like reaction could be an important mechanism of action of TDI.

The mouse, however, is also a good species to investigate IgE-dependent airway responses. In our laboratory Hessel and colleagues (116) developed a murine model to investigate human allergic asthma. Human allergic asthma is characterized by airway hyperresponsiveness to bronchoconstrictive mediators (222), the presence of allergen-specific IgE antibodies (55) and an influx of inflammatory cells, mainly eosinophils (282). In the mouse sensitization with ovalbumin (7 i.p. injections on alternate days) induced high ovalbumin-specific IgE levels in the serum. Moreover, repeated inhalation challenge with ovalbumin induced a significant *in vivo* airway hyperresponsiveness, which is also described by other investigators, and an influx of eosinophils in the lung (44, 255). In the present study we tried different sensitization regimes to induce the production of TDI-specific IgE antibodies. Furthermore, we investigated whether the presence of TDI-specific IgE antibodies had an influence on the airway reactivity, using *in vitro* (97) and *in vivo* techniques (117).

MATERIALS AND METHODS

Animals

Mice (male BALB/c 6-8 weeks of age) were supplied either by the Central Animal Laboratory, Utrecht, The Netherlands or by the National Institute of Public Health and the Environment, Bilthoven, The Netherlands. They were housed in groups not exceeding 6 per cage and maintained under standard conditions. All experiments were assessed by the animal ethics committee at Utrecht University and the National Institute of Public Health and the Environment.

Sensitization procedures

Short (2 day) exposure: Mice were sensitized twice daily on day 0 and day 1 either with 1% TDI (sensitized group) dissolved in acetone:olive oil (4:1) or with vehicle control (nonsensitized group) which was applied epicutaneously to the shaved abdomen and thorax (100 µl) and four paws (100 µl).

Long (6 weeks) exposure: Mice were sensitized once a day on day 0, 7, 14, 21, 28, and 35 (6 consecutive weeks) either with 1% TDI (sensitized group) dissolved in acetone:olive oil (4:1) or with vehicle control (nonsensitized group) which was applied epicutaneously to the shaved abdomen and thorax (100 µl) and four paws (100 µl). During all sensitization procedures the mice were anaesthetized with sodium pentobarbitone (50 µl; 30 mg/kg i.p.).

Challenge procedure

Sensitized and nonsensitized groups were challenged intranasally with 1% TDI dissolved in ethyl acetate:olive oil (1:4) either on day 8 (short exposure protocol) or on day 42 (long exposure protocol). Twenty µl of the TDI solution was applied intranasally under light anaesthesia (sodium pentobarbitone; 50 µl; 30 mg/kg i.p.). Furthermore, mice were also challenged on the ears; TDI (20 µl; 0.5%; dissolved in acetone) or vehicle control (20 µl) were applied topically to both sides of the ears.

Assessment of antibody production

Preparation TDI-MSA conjugates: 20 mg mouse serum albumin was added to 4 ml 0.05 M sodium phosphate buffer, pH 7.4. To this solution, 38 µl TDI was added. The mixture was stirred at 37°C for 4 hr. The reaction was stopped by addition of 5 µl monoethanolamine. After 1 hr, the solution was filtered and dialysed (273).

Enzyme-linked immunosorbent assay (ELISA): Polystyrene microtiter plates were coated with 50 µg/ml TDI-MSA for the TDI-specific IgE/IgG measurements or with 2,5 µg/ml rat anti-mouse IgE/IgG for the measurement of total IgE/IgG in 0.1 M carbonate buffer, pH 9.6 (50 µl per well). The coated plates were incubated in a humid chamber overnight at 4°C. Plates were washed five times with phosphate buffered saline (PBS)/Tween 20 (0.05% v/v) and incubated with 1% BSA in PBS/Tween 20 (0.05% v/v; 50 µl per well) for 30 minutes at room temperature. After washing the plates five times 50 µl diluted serum was added and incubated for 2.5 hr at room temperature. The plates were washed again five times and 50 µl of a 1:500 dilution of peroxidase labelled goat anti-mouse IgE/IgG was added. After a 2.5 hr incubation at room temperature, the plates were washed five

times and 50 µl o-phenylenediamine dihydrochloride (OPD) was added. After a further incubation period of 15 minutes at room temperature the reaction was stopped by adding 25 µl per well 4 M H_2SO_4 and absorbance values were read at 492 nm using a microplate reader. Values are given as optical density by a dilution of 200, 100, 1 x 107, 3200 for the total IgE, TDI-specific IgE, total IgG and TDI-specific IgE respectively. Sera samples were tested after the short exposure protocol (skin sensitization on 2 consecutive days and intranasal challenge on day 8), after the long exposure protocol (skin sensitization on 6 consecutive weeks and intranasal challenge on week 7) and during the long exposure protocol (skin sensitization on 4 consecutive weeks).

Measurement of *in vivo* airway responsiveness

In vivo airway responsiveness was measured using an air-overflow pressure method described by Hessel and coworkers (117). Mice were anaesthetized with urethane (2 g/kg i.p.) and placed on a heated blanket (30°C). The trachea was cannulated and a small polyethylene catheter was placed in the jugular vein for i.v. administrations. The spontaneous breathing was suppressed by tubocurarine chloride (3.3 mg/kg i.v.). Immediately hereafter the tracheal cannula was attached to a Fleisch flow head (Godart, Utrecht, The Netherlands), which was connected to a Gould Godart pneumotachograph (Godart, Utrecht, The Netherlands), which in turn was coupled to a respiration pump (Sanders Brinie, Enschede, The Netherlands). The inflation volume of the pump was 0.8 ml per beat with 190 beats per minute. A pressure transducer (Validyne, Norhtridge, CA, USA) was located between the flow head and the respiration pump in order to measure changes in the bronchial resistance to inflation. Pressure and flow signal were recorded breath-by-breath on a Gould Bruch 2400 recorder (Godart, Utrecht, The Netherlands). To asses reactivity dose-response curves to serotonin (20-1280 µg/kg i.v.) were determined 3, 24 and 48 hr after the challenge with TDI following both the long and short exposure protocol. Determination of doses-response curves to carbachol were not possible due to high mucus production leading to the death of the animals before the curves could be completed.

Isometric measurement of *in vitro* tracheal reactivity:

Tracheal reactivity was measured using the method of Garssen and coworkers (97). Mice were killed by an overdose of sodium pentobarbitone (0.3 ml; 60 mg/kg i.p.). The trachea, which was resected *in toto*, was carefully cleaned of connective tissue using a binocular microscope. A 9 ring piece of the trachea (taken from just below the larynx) was then transferred to a 10 ml organ bath containing a modified oxygenated Krebs solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCL_2 6H_2O$, 0.5 mM $MgCl_2 6H_2O$, 25.0 mM $NaHCO_3$, 1.0 mM $NaHPO_4 H_2O$, 11.1 mM glucose). The trachea was directly slipped onto 2 supports of an organ bath one of which was coupled to the organ bath and the other to an isometric transducer. The solution was aerated (95%: 5%; O_2: CO_2) at a constant temperature (37°C). Isometric measurements were made using a force displacement transducer (Harvard Bioscience, Boston, MA) and a 2 channel recorder (Servogar type SE-120) and were expressed as changes in mg force. Optimal pre-load for the mouse trachea was determined to be 1000 mg. The trachea was allowed to equilibrate for at least 1 hr before drug effects were elicited. During the equilibrium phase the fluid in the bath was changed every 15

minutes. To assess reactivity concentration-response curves to carbachol (10^{-8}-10^{-4} M) and serotonin (10^{-8}-10^{-4} M) were determined 2-3, 24 and 48 hr after challenge in both the long and short exposure protocol.

Histological analysis

At 0.5, 2 and 24 hr after and before the intranasal challenge on day 42 lungs and trachea were removed from mice after lethal anaesthesia with 50 µl of a cocktail consisting of 7 ml of 50 mg/ml ketalar, 3 ml 2% rompun, and 1 ml of 1 mg/ml atropine, injected intramuscularly (Nembutal causes vasodilatation, which negatively influences the histology preparations). Before removing the lungs the mice were perfused with 5 ml PBS (37°C) in the right heart ventricle. The lungs and trachea were filled intratracheally with a fixative (0.8% formalin, 4% acetic acid) using a ligature around the trachea. The unfolded lungs and trachea were fixed for at least 24 hr in the fixative, dehydrated, and embedded in paraplast. Four µm thick sections were stained with haematoxylin and eosin or with periodic acid Schiff reagent in combination with toluidine blue. An additional giemsa staining was performed to investigate the presence of eosinophils. Evaluation of the number of inflammatory cells and other histological changes was performed.

Measurement of cutaneous reactions

An increase in ear thickness was measured 2, 24 and 48 hr after topical challenge with 0.5% TDI in acetone after the short and long exposure protocol. Immediately after an i.p. overdose of sodium pentobarbitone the thickness of the TDI-treated ear and the vehicle-treated ear were measured using an engineers micrometer (Mitutoyo, Japan, No. 293-561) (48). Results are expressed as the difference in ear thickness (Δ ear thickness, mm) between the two ears.

Chemicals

Toluene diisocyanate, olive oil, mouse serum albumin, carbachol, serotonin (5-hydroxy tryptamine), o-phenylenediamine dihydrochloride, were purchased from Sigma Chemical Co. St. Louis, U.S.A. Rat anti-mouse IgE, rat anti-mouse IgG, rat anti-mouse IgE-peroxidase labelled, rat anti-mouse IgG-peroxidase labelled were purchased from Monosan. Tween 20 was purchased from Janssen Pharmaceutical, Belgium. Monoethanolamine was purchased from Merck, Amsterdam, The Netherlands. Sodium pentobarbitone was purchased from Sanofi, Maassluis, The Netherlands.

Statistics

All experiments were designed as completely randomized multifactorials with 6-14 mice per group. EC50-and Emax-values for the carbachol-induced tracheal contractions of each experimental animal were calculated separately by nonlinear least-squares regression analysis (simplex minimalization) of the measured contractions vs. carbachol concentration using the sigmoid concentration-response relationship and including a threshold value. The data were analysed by two-way Analysis of Variance followed by a post-hoc comparison between groups. In the figures and tables group means ± sem are given and a difference was considered significant when $P<0.05$. All data manipulation, non-linear fittings, Analyses of Variance and post-hoc comparisons were carried out with a commercially

a ailable statistical package (SYSTAT, version 5.03; Wilkinson L. SYSTAT: The system for statistics. Evanston, IL: SYSTAT, Inc., 1990. Statistics).

RESULTS

Antibody responses

Antibody levels were measured in TDI-sensitized mice and nonsensitized mice following the short exposure rotocol (sensitization twice daily on day 0 and day 1), the long exposure protocol (sensitization on 6 consecutive weeks followed by intranasal challenge on week 7) and after 4 consecutive weeks of sensitization. In the sera of TDI-sensitized and challenged mice following the short exposure protocol no increases in total IgE, TDI-specific IgE and total IgG were found while an increase in TDI-specific IgG was detected when compared with the nonsensitized and challenged mice (*figure 1A-D*). However, sensitization on 6 consecutive weeks and challenge on week 7 caused an increase in all antibodies measured. *Figure 1A and C* show that both the total IgE and the total IgG levels were significantly increased in the sera of TDI-sensitized and challenged mice when compared with nonsensitized mice following the long exposure protocol ($P<0.01$ and $P<0.05$ respectively). More importantly, TDI-specific IgE and TDI-specific IgG were also significantly elevated in the sera of TDI-sensitized and challenged mice when compared with the control mice ($P<0.01$, *figure 1B and D*). The formation of antibodies in time was followed by measuring the levels after sensitization for 4 consecutive weeks. After TDI-sensitization on 4 consecutive weeks the total IgE level had already increased to the same level as after 6 weeks of sensitization and challenge on week 7 (*figure 1A*). In contrast, the TDI-specific IgE was only 57% of the increase found after sensitization on 6 consecutive weeks and challenge on week 7 (*figure 1B*).

In vivo airway responsiveness

In vivo airway responsiveness to serotonin was measured in TDI-sensitized and nonsensitized mice both after the short and long exposure protocol. *Figure 2* displays typical dose-response curves to serotonin of a TDI-sensitized and a nonsensitized mouse 3 hr after the challenge following the long exposure protocol to illustrate that injection of serotonin (i.v.) resulted in a direct contraction of the airway smooth muscle. In *figure 3* the *in vivo* airway reactivity to serotonin 3 and 24 hr after the challenge of TDI-sensitized mice and nonsensitized mice following the long exposure protocol are depicted. Three hr after

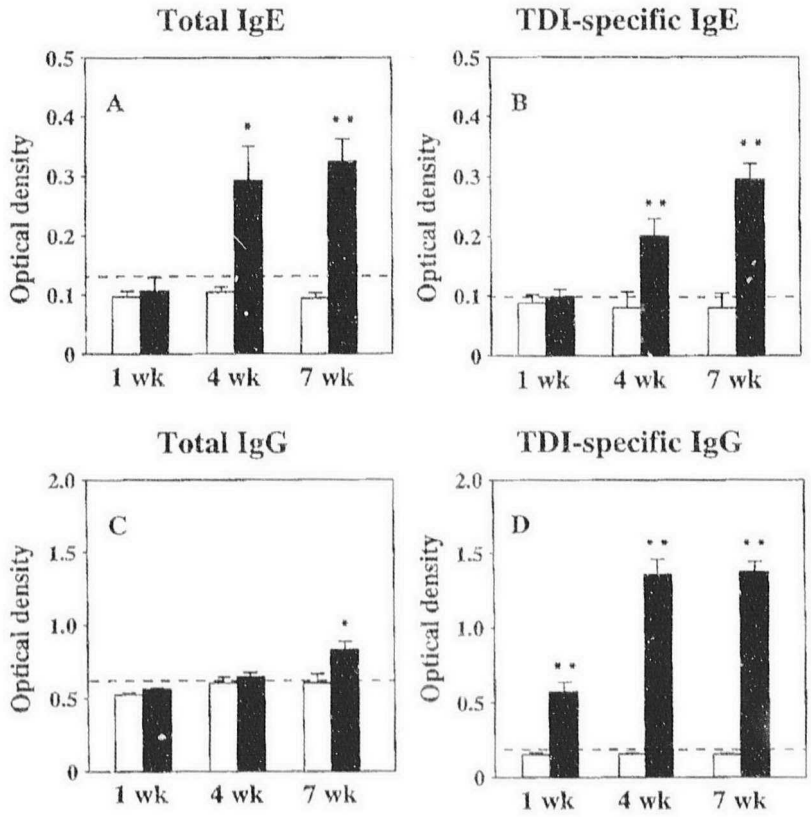

Figure 1 *Antibody levels in sera of TDI-sensitized (closed bars) and nonsensitized (open bars) mice (open bars). (A) Total IgE, (B) TDI-specific IgE, (C) total IgG and (D) TDI-specific IgG were determined by spectrophotometry in sera of mice after the short exposure protocol (sensitization on day 0 and day 1 followed by challenge on day 8 (1 wk)), after the long exposure protocol (sensitization on 6 consecutive weeks followed by challenge on week 7 (7 wk)) and after sensitization of 4 consecutive weeks (4 wk). The dotted lines represent the respective antibody levels in the sera of naïve mice. Results are expressed as mean ± sem for n=4 mice/group. Significant differences between TDI-sensitized and nonsensitized mice ($P<0.05$ and $P<0.01$) are denoted by (*) and (**) respectively.*

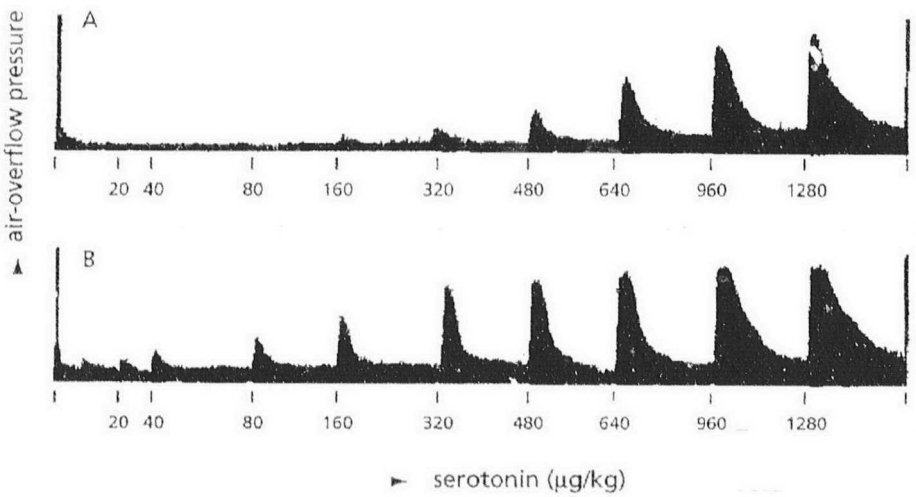

Figure 2 Typical traces of in vivo serotonin doses-response curves (20-1280 µg/kg i.v.) in a (A) nonsensitized and (B) TDI-sensitized mouse 3 hr after the challenge following the long exposure protocol. In vivo airway reactivity to serotonin was measured by an air-overflow pressure method described by Hessel and coworkers (117).

the challenge on day 42 the TDI-sensitized group was clearly more sensitive to serotonin when compared with the nonsensitized group (*figure 3A*). The increase in airway hyperresponsiveness was significant at the doses of 20-480 µg/kg serotonin ($P<0.05$). This hyperresponsiveness could not be found when mice were skin sensitized on 6 consecutive weeks with TDI but not challenged on day 42 (data not shown). *Figure 3B* indicates that at 24 hr after the challenge this *in vivo* airway hyperresponsiveness to serotonin of TDI-sensitized mice following the long exposure protocol had disappeared. *In vivo* airway reactivity was also measured after TDI-sensitization following the short exposure protocol. In contrast to the long exposure protocol, no difference in responsiveness to serotonin between TDI-sensitized and the nonsensitized mice could be found 3 and 24 hr after the challenge on day 8 (*figure 4*). When the airway reactivity was tested 48 hr after the challenge there was also no difference between TDI and nonsensitized mice both after the long and the short exposure protocol (data not shown). In *table 1* the respective EC50

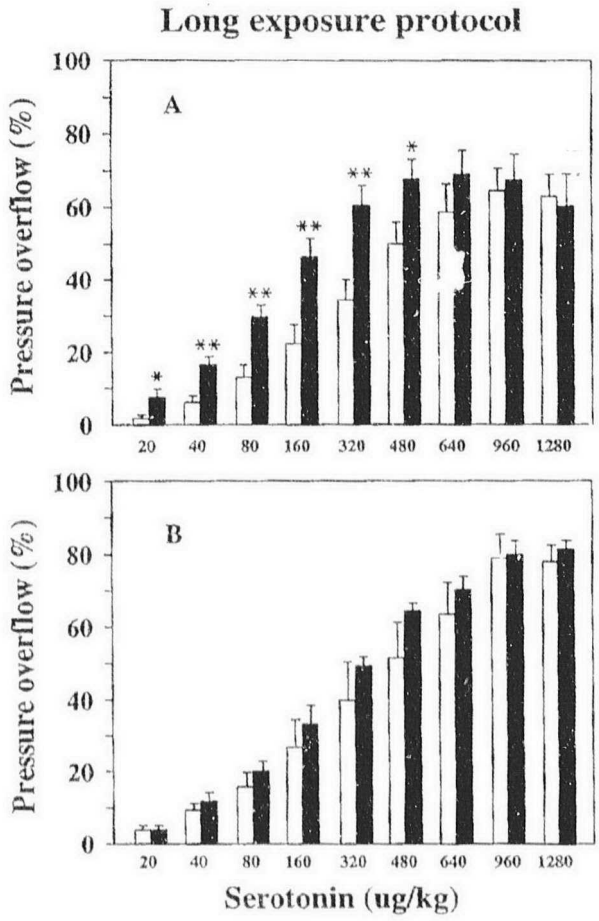

Figure 3 In vivo airway reactivity (A) 3 hr and (B) 24 hr after the challenge. Doses-response curves to serotonin were measured in TDI-sensitized (closed bars) and nonsensitized (open bars) mice 3 and 24 after the challenge on day 42 (according to the long exposure protocol). Results are expressed as mean ± sem for n=6-9 mice/group. Significant differences between TDI-sensitized and nonsensitized mice ($P<0.05$ or $P<0.01$) are denoted by (*) or (**) respectively.

(effective concentration that produced 50% of the response) values of TDI-sensitized and nonsensitized mice are listed. A significant shift in EC50 values could only be found 3 hr after the challenge in TDI-sensitized mice when compared with the nonsensitized mice following the long exposure protocol.

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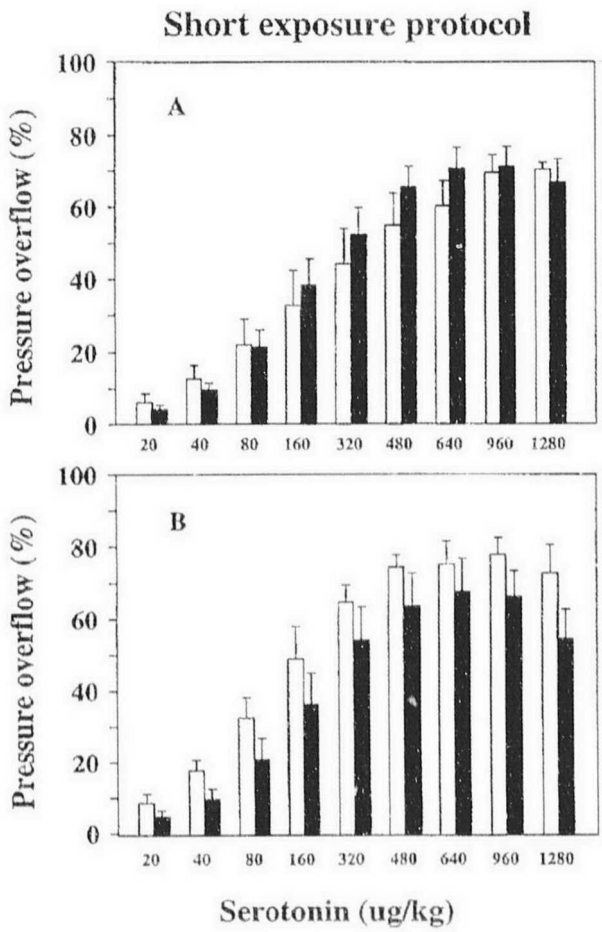

Figure 4 In vivo airway reactivity (A) 3 hr and (B) 24 hr after the challenge. Doses-response curves to serotonin were measured in TDI-sensitized (closed bars) and nonsensitized (open bars) mice 3 and 24 hr after the challenge on day 8 (according to the short exposure protocol). Results are expressed as mean ± sem for n=6 mice/group.

Table 1. ED50 (µg/kg) values to serotonin of TDI-sensitized and nonsensitized mice 3, 24 and 48 hr after the challenge in the long and short exposure protocol. *In vivo* airway reactivity to serotonin was measured by an air-overflow pressure method described by Hessel and coworkers (117).

Sensitization	Time after challenge		
	3 hr	24 hr	48 hr
Long exposure protocol			
Control	330 ± 63	369 ± 82	295 ± 44
TDI	115 ± 11*	217 ± 27	217 ± 48
Short exposure protocol			
Control	337 ± 71	151 ± 32	268 ± 80
TDI	231 ± 50	226 ± 67	267 ± 71

Results are expressed as mean ± sem for n=5-9 mice/group. Significant differences between TDI-sensitized and nonsensitized groups are denoted by (*) for $P<0.05$.

In vitro tracheal reactivity

To investigate the correlation between *in vitro* and *in vivo* parameters the tracheal reactivity *in vitro* was also measured. *Figure 5A* shows the tracheal reactivity to carbachol 3 hr after the challenge on day 42 (long exposure protocol). The TDI-sensitized mice were hyperreactive to carbachol when compared with the nonsensitized mice (Emax: nonsensitized 2247 ± 146 mg; TDI-sensitized 3019 ± 154 mg, mean ± sem for n=6 mice/group, $P<0.05$). Twenty four and 48 hr after the challenge no difference in tracheal hyperreactivity to carbachol between the TDI-sensitized and nonsensitized mice was found (Emax, 24 hr after the challenge: nonsensitized 2100 ± 301 mg; TDI-sensitized 2347 ± 151 mg. Emax, 48 hr after the challenge: nonsensitized 2650 ± 124; TDI-sensitized 2765 ± 159 mg, mean ± sem for n=5-14 mice/group). In contrast to the concentration-response curves to carbachol, reproducible concentration-response curves to serotonin *in vitro* were difficult to obtain in naive and treated BALB/c mice. However, in each experiment after long term exposure to TDI the TDI-sensitized mice were more reactive to serotonin 3 hr after the challenge when compared with the nonsensitized mice (Emax experiment 1: nonsensitized 660 ± 230 mg; TDI-sensitized 2103 ± 426 mg. Experiment 2: nonsensitized 934 ± 193 mg; TDI-sensitized 1486 ± 375 mg. Experiment 3: nonsensitized 1921 ± 235 mg; TDI-sensitized 2389 ± 188 mg. Experiment 4: nonsensitized 2152 ± 299 mg; TDI-sensitized 2599 ± 224 mg, mean ± sem for n=4-6 mice/group in each experiment). Due to

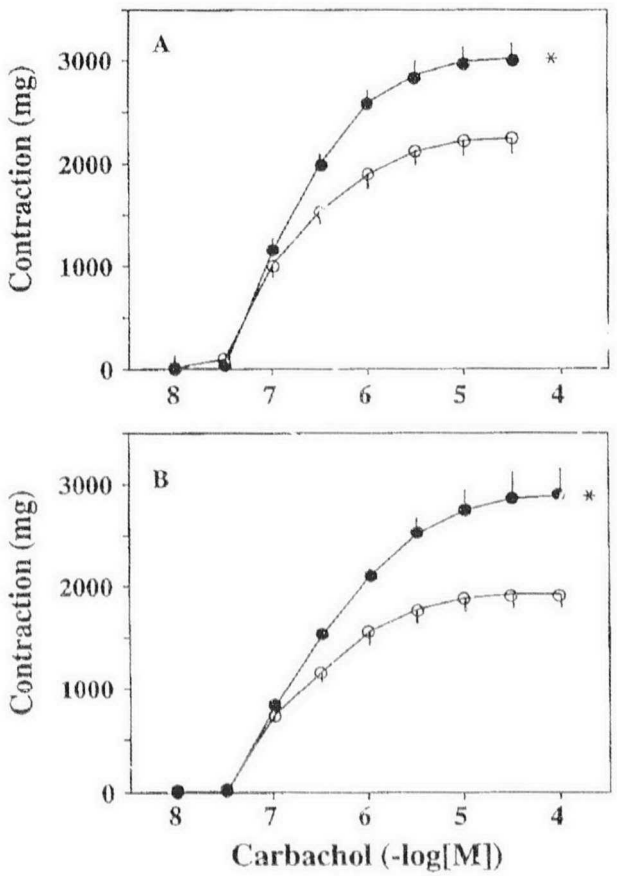

Figure 5 *Tracheal reactivity to carbachol (A) 2-3 hr after the challenge following the long exposure protocol and (B) 24 hr after the challenge following the short exposure protocol. Concentration-response curves to carbachol were measured in the trachea of TDI-sensitized (closed circles) and nonsensitized (open circles) mice. Results are expressed as mean ± sem for n=6-12 mice/group. Significant differences between TDI-sensitized and nonsensitized mice (P<0.05) are denoted by (*).*

the degree of variation following this procedure, the overall figures of EC50 or Emax values of the TDI-sensitized mice were not significantly different from the EC50 or Emax values of the nonsensitized mice. In comparison, a significant tracheal hyperreactivity to carbachol (*figure 5B*) and serotonin (Emax: nonsensitized 1837 ± 118 mg; TDI-sensitized 2430 ± 197 mg, mean ± sem for n=12 mice/group, P<0.05) were found in TDI-sensitized

mice 24 hr after the challenge after sensitization according to the short exposure protocol. At 2 and 48 hr there was no significant difference in tracheal reactivity between the TDI-sensitized and nonsensitized mice as previously described (274).

Histological analysis

Light microscopy was performed to investigate the effect of TDI sensitization and challenge according to the long exposure protocol on the airways. Skin sensitization on 6 consecutive weeks with 1% TDI did not result in any histological changes in the airways. However, the intranasal challenge with 1% TDI caused hypertrophy of the epithelium within 2 hr in both the TDI-sensitized and nonsensitized mice. Twenty four hr after the challenge necrosis of the bronchial epithelium was accompanied by mucus production and an infiltration of eosinophils in the epithelium and around the bronchioli resulting in total desquamation of the bronchial epithelium. However, the observed changes were due to the irritant effect of the intranasal challenge since they were apparent in the TDI-sensitized as well as in the nonsensitized mice.

Cutaneous responses

In addition to measuring pulmonary responses induced by long exposure to TDI the development of cutaneous immune responsiveness was followed. *Figure 6A* shows the increase in ear thickness 2-3, 24 and 48 hr after topical challenge with 0.5% TDI. At all time points measured the increase in ear thickness in the TDI-sensitized group after the long exposure protocol was significantly higher when compared with the nonsensitized group ($P<0.05$). In addition, the cutaneous responses of TDI-sensitized and nonsensitized mice following the short exposure protocol were examined. In comparison to the long exposure protocol it is striking that 2-3 hr after the challenge no increase in ear swelling could be measured in TDI-sensitized mice after the short exposure protocol when compared with the nonsensitized mice (*figure 6B*). However, 24 hr after the challenge a significant increase in ear swelling in TDI-sensitized mice was found when compared with the nonsensitized mice. This increase was largely resolved 48 hr after the challenge (*figure 6B*).

A summary of the presented data in TDI-sensitized and challenged mice following the short and the long exposure protocol is given in *table 2*.

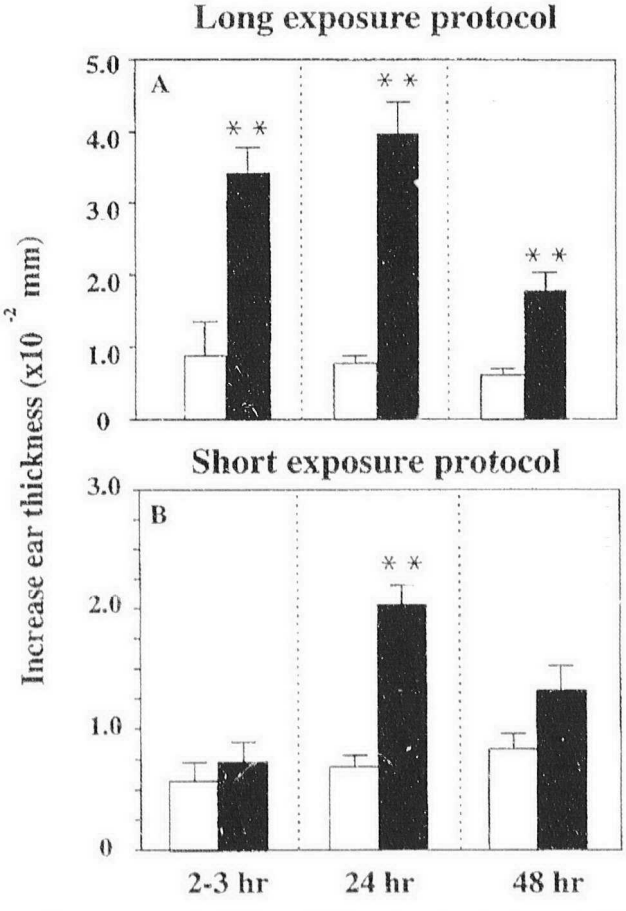

Figure 6 *Ear swelling response 2-3, 24 and 48 hr after the challenge in TDI-sensitized and nonsensitized mice following (A) the short exposure protocol and (B) the long exposure protocol. TDI-sensitized (closed bars) and nonsensitized (open bars) mice were topically challenged on day 8 (short exposure protocol) or on day 42 (long exposure protocol) on both ears; one ear with 0.5% TDI and the other with vehicle. The ear swelling was measured 2-3, 24 and 48 hr after the challenge using a micrometer and the difference in ear thickness (x 10^{-2} mm) between the two ears is expressed as mean ± sem for n=6 mice/group. Significant differences between bars (P<0.05 and P<0.01) are denoted by (*) and (**) respectively.*

Table 2. Summary of the presented data of TDI-sensitized mice following the short and long exposure protocol.

	Short exposure protocol	Long exposure protocol
TDI-specific IgE antibodies	-	+
In vitro tracheal hyperreactivity	24 hr	3 hr
In vivo airway hyperresponsiveness	-	3 hr
Histological changes	-	-
Cutaneous ear swelling	24 hr	3, 24, 48 hr

DISCUSSION

We have recently developed a murine model to investigate TDI-induced occupational asthma (274). Skin sensitization on day 0 and day 1 with 1% TDI and intranasal challenge on day 8 (1% TDI) led to *in vitro* tracheal hyperreactivity to carbachol and serotonin 24 hr after the challenge. This model was not associated with an increase in TDI-specific IgE antibodies and can therefore be used to investigate IgE-independent TDI-induced occupational asthma which accounts for as much as 80% of the reported patients. We hypothesized that skin sensitization with TDI according to the short exposure model resulted in a DTH-like reaction (274). DTH reactions are characterized by Th1 responses resulting in IgG2a, but not IgE synthesis. Accordingly, in the present study we have indeed found an increase in TDI-specific IgG and not in IgE antibodies after TDI application following the short exposure protocol. In the remaining 20% of the subjects with TDI-induced occupational asthma IgE antibody production does seem to play an important role in the development of occupational asthma. In this present study we developed a new sensitization protocol for TDI which resulted in TDI-specific IgE antibody production. Recently, Kitagaki and coworkers (143) demonstrated that repeated application of contact sensitizing agents to the same skin site resulted in a shift in the time course of antigen-

specific hypersensitivity responses from a typical DTH to an immediate-type reaction followed by a late reaction. Interestingly, in our study repeated application of TDI for 6 consecutive weeks did lead to an increase in total and TDI-specific IgE antibodies which, in general, are associated with an immediate-type response. In addition, both total and TDI-specific IgG were increased, suggesting the simultaneous induction of IgG1 antibodies.

Varying the exposure regime to TDI did not only result in different antibody responses, but the development of airway responses was also differentially affected. TDI sensitization according to the short exposure protocol resulted in *in vitro* tracheal hyperreactivity 24 hr after the challenge whereas no *in vivo* airway changes were observed. The reason for the discrepancy between *in vitro* and *in vivo* airway reactivity with regard to the short exposure protocol could be that the *in vitro* tracheal reactivity method might be more sensitive when compared with the *in vivo* airway reactivity technique. Additionally, using the *in vitro* technique a specific isolated peace of trachea is taken and consequently reactivity of the bronchi and any other influences from the intact mouse, which are still present when *in vivo* airway changes are measured, could by negated. In contrast, the long exposure protocol caused more immediate changes in airway parameters. Indeed, three hr after the challenge on day 42 (i.e. after the long exposure protocol) *in vitro* tracheal hyperreactivity to carbachol and serotonin were found in TDI-sensitized mice when compared with nonsensitized mice. Moreover, TDI sensitization following the long exposure protocol resulted in *in vivo* airway hyperresponsiveness 3 hr after the challenge.

The dissimilarity between the IgE-mediated (long exposure) and IgE-independent (short exposure) mechanism was also obvious when cutaneous responses were measured. TDI sensitization according to the long exposure protocol resulted in a significant ear swelling response 2-3 hr after the challenge which was not found after TDI sensitization according to the short exposure protocol. Taken together, TDI sensitization and challenge according to the long exposure protocol resulted in the production of TDI-specific IgE antibodies accompanied by more immediate reactions in the airways and skin when compared with sensitization according to the short exposure protocol. Although so far no relationship between IgE or IgG antibodies to TDI and the respiratory response in asthmatics sensitized to isocyanates has been reported, it was suggested that the early-onset responses reflected an IgE-mediated response (140). The study of Karol and coworkers, in addition to the data presented here, demonstrate that TDI is capable of inducing different immunological reactions. We hypothesize that the short exposure protocol resembles the IgE-independent mechanism of action of TDI and that the long exposure protocol is a viable model to investigate IgE-mediated TDI-induced occupational asthma. In future studies, the strict

IgE-dependency of the airway hyperresponsiveness observed in this model will have to be established.

In conclusion, this study demonstrated that by altering the exposure time and/or cumulative dosage TDI is capable of inducing different immunological reactions. This important finding might be an explanation for the diversity of symptoms found in patients suffering from TDI-induced asthma. Both the short exposure and the long exposure model will be useful to further investigate the mechanisms of action of TDI.

CHAPTER 4

The involvement of sensory neuropeptides in toluene diisocyanate-induced tracheal hyperreactivity in the mouse airways

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The involvement of sensory neuropeptides in toluene diisocyanate-induced tracheal hyperreactivity in the mouse airways

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ABSTRACT

Recently, we have developed a murine model to investigate toluene diisocyanate (TDI)-induced occupational asthma. After skin sensitization and intranasal challenge with TDI (1%) mice exhibited tracheal hyperreactivity 24 hr after the challenge. The aim of this present study was to investigate the possible role for sensory neuropeptides in the development of this tracheal hyperreactivity. First, we demonstrated that direct application of TDI *in vitro* induced the release of tachykinins from the sensory nerves in the isolated mouse trachea. Second, capsaicin pretreatment, resulting in the depletion of sensory neuropeptides, completely abolished the TDI-induced tracheal hyperreactivity 24 hr after the challenge. Third, the selective neurokinin $_1$ (NK_1) receptor antagonist RP 67580 (0.2 µmol/kg, i.v.) also inhibited tracheal hyperreactivity when it was administered before the challenge. Administration of RP 67580 during the sensitization phase, however, did not result in a suppression of the TDI-induced tracheal hyperreactivity 24 hr after the challenge. When TDI-sensitized mice were topically challenged with TDI a marked ear swelling response was observed. The cutaneous response after TDI application was not affected by capsaicin pretreatment or RP 67580 administration.

These results clearly show that sensory neuropeptides, particularly tachykinins, are essential for the development of TDI-induced tracheal hyperreactivity during the effector phase. The differences between the airways and skin with respect to the sensory neuropeptides are intriguing and could suggest a local action for the tachykinins in the airways.

INTRODUCTION

Toluene diisocyanate (TDI), a low molecular weight compound, is a well known cause of occupational asthma (27). Sensitized subjects exhibit specific airway hyperresponsiveness and a marked inflammation of the airways, characterized by an influx of neutrophils and eosinophils (34, 178). The mechanisms underlying these symptoms are controversial. In only 20% of the subjects with TDI-induced asthma an increase in TDI-specific IgE antibodies has been reported (51). Recently, we have developed a murine model to investigate the mechanisms of action of TDI (274). After skin sensitization on two consecutive days and intranasal challenge 7 days later with 1% TDI no increase in serum IgE was observed. However, 24 hr after the challenge a marked increase in tracheal

reactivity to carbachol was measured in TDI-sensitized mice when compared with nonsensitized mice.

The sensory nerves, which are part of the noncholinergic, nonadrenergic (NANC) system, are thought to play an important role in the skin and airways. In the respiratory tract, sensory nerves are found in abundance around pulmonary blood vessels and in the epithelium of the trachea and bronchi of many species (18, 105). They contain a range of neuropeptides including the tachykinins (substance P (SP) and neurokinin A (NKA)) (166) and calcitonin gene-related peptide (CGRP) (172). The tachykinins can induce bronchoconstriction via activation of the NK_2 receptor (164) and mucus production (259), plasma protein leakage, and vasodilatation (167) in the airways via the activation of NK_1 receptors. However, in mice airways only NK_1 receptors have been demonstrated (174). Additionally, SP has been demonstrated to degranulate mast cells by a non-receptor mediated mechanism, thus promoting the release of vasoactive amines such as histamine and serotonin (19, 82). The most important biological action of CGRP is the regulation of airway blood flow; it is a potent vasodilator (39). Interestingly, several studies have already demonstrated that TDI is capable of inducing the release of sensory neuropeptides from capsaicin-sensitive nerves *in vivo* and *in vitro* (60, 144, 180, 182).

More recent data have revealed the ability of various sensory neuropeptides to modulate immune functions. The tachykinins have been reported to induce proliferation of mitogen-stimulated T lymphocytes (56, 234, 287), migration of leukocytes (187, 207), and activation of macrophages (145). In contrast, CGRP has been reported to inhibit the proliferation of mitogen-stimulated T lymphocytes (56, 301), although several reports also suggest that CGRP may be proinflammatory of its own (45, 46, 92). In literature, the role for sensory neuropeptides in the induction of airway hyperreactivity has been investigated in several studies (32, 267). In addition, studies in our own laboratory have recently demonstrated the importance of sensory neuropeptides in a murine model for pulmonary delayed-type hypersensitivity (DTH) reaction (47). The aim of the present study was to investigate the role for sensory nerves and the tachykinins in the sensitization and effector phase of TDI-induced tracheal hyperreactivity in the mouse.

MATERIALS AND METHODS

Animals

Mice (male BALB/c 6-8 weeks of age) were supplied either by the Central Animal Laboratory, Utrecht, The Netherlands or by the National Institute of Public Health and the

Environment, Bilthoven, The Netherlands. They were housed in groups not exceeding 6 per cage and maintained under standard conditions. All experiments were assessed by the animal ethics committee at Utrecht University and the National Institute of Public Health and the Environment.

Isometric measurement of tracheal reactivity

Tracheal reactivity was measured using the method of Garssen and coworkers (97). Mice were killed by an overdose of sodium pentobarbitone (0.3 ml; 60 mg/kg i.p.). The trachea, which was resected *in toto*, was carefully cleaned of connective tissue using a binocular microscope. An intact piece of 9 tracheal rings (taken from just below the larynx) was then transferred to a 10 ml organ bath containing a modified oxygenated Krebs solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCL_2.6H_2O$, 0.5 mM $MgCl_2.6H_2O$, 25.0 mM $NaHCO_3$, 1.0 mM $NaHPO_4.H_2O$, 11.1 mM glucose). The trachea was directly slipped onto 2 supports of an organ bath one of which was coupled to the organ bath and the other to an isometric transducer. The solution was aerated (95%: 5%; O_2: CO_2) at a constant temperature (37°C). Isometric measurements were made using a force displacement transducer (Harvard Bioscience, Boston, MA) and a 2 channel recorder (Servogar type SE-120) and were expressed as changes in mg force. Optimal pre-load for the mouse trachea was determined to be 1000 mg. The trachea was allowed to equilibrate for at least 1 hr prior to further manipulations. During the equilibrium phase the fluid in the bath was changed every 15 minutes. To assess reactivity concentration-response curves to carbachol or serotonin were determined 24 hr after challenge with TDI. In some experiments the direct effect of TDI and substance P on the isolated mouse trachea was measured *in vitro*. After precontraction with carbachol (3×10^{-7} M bath concentration) one single concentration of TDI (ranging from 10^{-6}-10^{-4} M) or substance P (ranging from 10^{-9}-10^{-6} M) was added to the organ bath. TDI was dissolved in dimethyl sulphoxide (DMSO); however, the concentration of DMSO did not exceed 0.1% in the organ bath and had no effect on its own. To study the effect of RP 67580, the trachea was incubated for 30 minutes with RP 67580 (10^{-8}-10^{-6} M bath concentrations) before precontraction with carbachol and subsequent addition of TDI or substance P. In separate experiments, isoprenaline (10^{-7} M bath concentration), which is also an relaxant on murine smooth muscle, was added to the precontracted trachea to establish the specificity of RP 67580 for the NK_1 receptor.

Sensitization procedures

Mice were sensitized twice daily on day 0 and day 1 either with 1% TDI (sensitized group) dissolved in acetone:olive oil (4:1) or with vehicle control (nonsensitized group) which was applied epicutaneously to the shaved abdomen and thorax (100 µl) and four paws (100 µl). During the sensitization procedure the mice were anaesthetized with sodium pentobarbitone (50 µl; 30 mg/kg i.p.).

Challenge procedure

TDI-sensitized and nonsensitized mice were challenged with 1% TDI dissolved in ethyl acetate:olive oil (1:4) on day 8. Twenty µl of the TDI solution was applied intranasally under light anaesthesia (sodium pentobarbitone; 50 µl; 30 mg/kg i.p.). Furthermore, mice

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Statistics

All experiments were designed as completely randomized multifactorials with 6-14 mice per group. EC50-and Emax-values for the carbachol-induced tracheal contractions of each experimental animal were calculated separately by nonlinear least-squares regression analysis (simplex minimalization) of the measured contractions vs. carbachol or serotonin concentration using the sigmoid concentration-response relationship and including a threshold value. The data were analysed by two-way Analysis of Variance followed by a post-hoc comparison between groups. In the figures and tables group means ± sem are given and a difference was considered significant when $P<0.05$. All data manipulation, non-linear fittings, Analyses of Variance and post-hoc comparisons were carried out with a commercially available statistical package (SYSTAT, version 5.03; Wilkinson L. SYSTAT: The system for statistics. Evanston, IL: SYSTAT, Inc., 1990. Statistics).

RESULTS.

Direct effect of TDI and substance P on the isolated mouse trachea

Tracheal preparations taken from naive mice were tested for their reactivity to directly applied TDI and SP. TDI (10^{-4} M) and SP (10^{-7} M) had no effect on the resting basal tone of the isolated mouse trachea. However, after precontraction with carbachol (3×10^{-7} M bath concentration) TDI produced a concentration-dependent relaxation of the mouse trachea with a maximal effect at 10^{-4} M bath concentration (*figure 1A*). A similar concentration-dependent relaxation was found after administration of SP (3×10^{-10}-3×10^{-7} M bath concentration) to the precontracted trachea (*figure 1B*). In further experiments the effect of the NK_1 receptor antagonist, RP 67580, on the relaxation induced by TDI and SP was examined. Incubation with RP 67580 for 30 minutes did not have any effect on the precontraction induced by carbachol. *Figures 2A* and *2B* show that the relaxation induced by TDI (10^{-5} M) and SP (3×10^{-9} M) were both concentration-dependently inhibited by RP 67580 (10^{-8} - 10^{-6} M), with a maximal effect at 10^{-6} M (90% inhibition). To demonstrate the specificity for the NK_1 receptor, we tested the effect of RP 67580 on isoprenaline-induced relaxation. RP 67580 (10^{-6} M) had no effect on isoprenaline (10^{-7} M)-induced relaxation of the isolated mouse trachea (isoprenaline: 92 ± 3 % relaxation; isoprenaline + RP 67580: 94 ± 2 % relaxation, mean ± sem for n=6 mice/group). Moreover, capsaicin pretreatment completely inhibited TDI-induced relaxation, whereas SP-induced relaxation was unaltered (*table 1*).

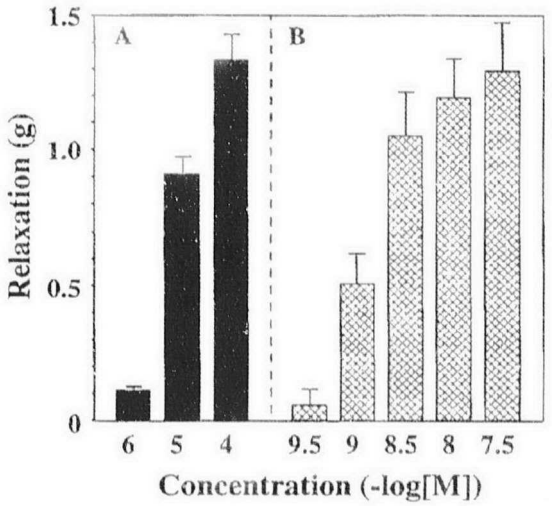

Figure 1 Direct effect of (A) toluene diisocyanate (TDI) and (B) substance P on the isolated mouse trachea. Tracheal preparations were first precontracted with carbachol (3 x 10^{-7} M bath concentration) where after TDI (10^{-6}-10^{-4} M) or substance P (3 x 10^{-10}-3 x 10^{-7} M) were added. Results are expressed as mean ± sem for n=4-6 mice/group.

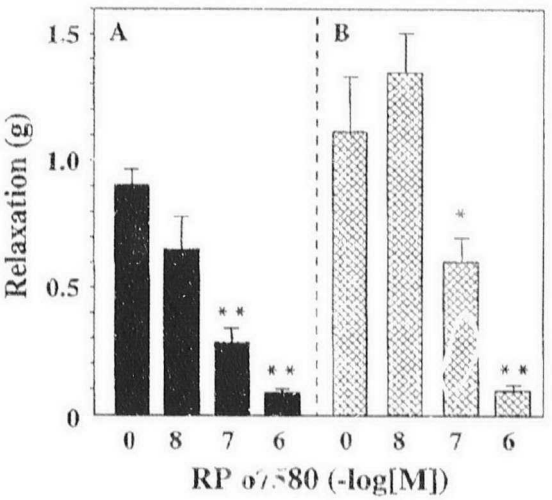

Figure 2 Effect of RP 67580 on the (A) toluene diisocyanate (TDI) and (B) substance P-induced tracheal relaxation. Before precontraction with carbachol (3 x 10^{-7} M bath concentration) and subsequent relaxation with TDI (10^{-5} M) or substance P (3 x 10^{-9} M) the tracheal preparations were incubated for 30 minutes with RP 67580 (10^{-8} - 10^{-6} M). Results are expressed as mean ± sem for n=4-6 mice/group. Significant differences between bars are denoted by (*) and (**) (P<0.05 and P<0.01 respectively).

Table 1. Effect of capsaicin pretreatment on TDI- and SP-induced relaxation in the isolated mouse trachea.

Pretreatment	Relaxing agent	% relaxation
Vehicle	Capsaicin	65 ± 6
Capsaicin	Capsaicin	20 ± 5**
Vehicle	Substance P	67 ± 8
Capsaicin	Substance P	66 ± 8
Vehicle	TDI	73 ± 3
Capsaicin	TDI	12 ± 6**

Results are expressed as mean ± sem for n=4-7 mice/group. Significant differences are denoted by (**) for $P<0.01$ when compared with the vehicle-pretreated mice.

Effect of capsaicin pretreatment on tracheal hyperreactivity

First, the effectiveness of sensory neuropeptide depletion by capsaicin was assessed by adding capsaicin (100 nM) to trachea precontracted with carbachol (3×10^{-7} M bath concentration). Direct application of capsaicin induced a relaxation of 17 ± 4% in capsaicin-pretreated mice in comparison to a 60 ± 8% relaxation in vehicle-pretreated mice. These results are in agreement with previous studies where depletion of sensory neuropeptides was tested in the airways and the skin (47). Once sensory neuropeptide depletion had been established, tracheal preparations taken from TDI-sensitized and nonsensitized mice, pretreated with capsaicin or vehicle, were tested for their reactivity to carbachol and serotonin (10^{-8} - 10^{-4} M) 24 hr after intranasal challenge with TDI. Twenty four hr after the challenge the vehicle-pretreated TDI-sensitized mice exhibited hyperreactivity both to the chemically dissimilar compounds carbachol and serotonin when compared with the vehicle-pretreated nonsensitized mice (*figure 3, table 2*). TDI-induced tracheal hyperreactivity to carbachol was completely inhibited by capsaicin pretreatment (*figure 3, table 2*). Interestingly, capsaicin pretreatment itself increased the tracheal reactivity to carbachol in nonsensitized mice, however not significantly (*figure 3, table 2*). The sensitivity (EC50) to carbachol and serotonin remained unchanged in all groups tested (*table 2*).

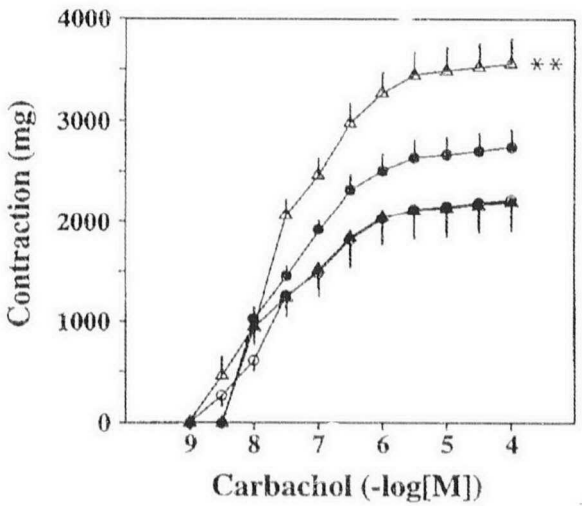

Figure 3 Tracheal reactivity after capsaicin pretreatment in TDI-sensitized and nonsensitized mice. Concentration-response curves to carbachol were measured in the trachea of nonsensitized (circles) and TDI-sensitized (triangles) mice either pretreated with capsaicin (closed symbols) or vehicle (open symbols) 24 hr after intranasal challenge with 1% TDI. Results are expressed as mean ± sem for n=6 mice/group. Significant differences (P<0.01) between the vehicle-pretreated-TDI-sensitized group and the capsaicin-pretreated-TDI-sensitized groups are denoted by (**).

Table 2. EC50 and Emax values derived from concentration-response curves to carbachol and serotonin (10^{-8}-10^{-4} M) in nonsensitized and TDI-sensitized mice after vehicle or capsaicin pretreatment.

Pretreatment	Sensitization	Contractile agent	EC50 (10^{-7} M)	Emax (mg)
Vehicle	Control	Carbachol	0.274 ± 0.041	2225 ± 266
Vehicle	TDI	Carbachol	0.263 ± 0.044	3564 ± 240*
Vehicle	Control	Serotonin	3.76 ± 1.05	1837 ± 118
Vehicle	TDI	Serotonin	2.53 ± 0.27	2430 ± 197*
Capsaicin	Control	Carbachol	0.215 ± 0.024	2630 ± 171
Capsaicin	TDI	Carbachol	0.352 ± 0.192	2115 ± 280

Results are expressed as mean ± sem for n=6 mice/group. Significant differences are denoted by (*) for P<0.05 between the nonsensitized and TDI-sensitized mice.

Table 3. EC50 and Emax values derived from concentration-response curves to carbachol (10^{-3}-10^{-4} M) in nonsensitized and TDI-sensitized mice after RP 67580 treatment.

Sensitization	Treatment	Administration (day)	EC50 (x 10^{-7} M)	Emax (mg)
Control	RP 68651	0-1-8	0.364 ± 0.052	2248 ± 263
TDI	RP 68651	0-1-8	0.339 ± 0.164	3120 ± 298*
Control	RP 67580	0-1-8	0.398 ± 0.062	2148 ± 85
TDI	RP 67580	0-1-8	0.345 ± 0.098	2223 ± 133¶
Control	RP 68651	0-1	0.409 ± 0.065	2160 ± 183
TDI	RP 68651	0-1	0.504 ± 0.095	2770 ± 131*
Control	RP 67580	0-1	0.500 ± 0.125	2279 ± 143
TDI	RP 67580	0-1	0.674 ± 0.116	2770 ± 131*
Control	RP 68651	8	0.324 ± 0.065	1886 ± 139
TDI	RP 68651	8	0.554 ± 0.351	2930 ± 238**
Control	RP 67580	8	0.277 ± 0.050	2186 ± 235
TDI	RP 67580	8	0.273 ± 0.062	2235 ± 226

Results are expressed as mean ± sem for n=4-8 mice/group. Significant differences are denoted by (*) or (**) for $P<0.05$ or $P<0.01$ respectively between the nonsensitized and TDI-sensitized mice or by ¶ for $P<0.05$ between the RP 68651 and RP 67580-treated mice.

Effect of NK_1 receptor antagonist on tracheal hyperreactivity

The effects of RP 67580, the specific NK_1 receptor antagonist and its inactive enantiomer RP 68651 were measured both during the sensitization and effector phases on the TDI-induced tracheal hyperreactivity 24 hr after intranasal challenge with TDI. RP 67580 (0.2 µmol/kg, i.v.) had no effect on the TDI-induced tracheal hyperreactivity when it was administered 5 minutes before each application of TDI during the sensitization phase (*table 3*). However, when RP 67580 (0.2 µmol/kg, i.v.) was administered 5 minutes prior to the TDI challenge, tracheal hyperreactivity was suppressed and there was no significant difference between the TDI-sensitized and nonsensitized mice (*table 3*). Furthermore, RP 67580 (0.2 µmol/kg, i.v.) abolished the TDI-induced tracheal hyperreactivity when administered 5 minutes before each application of TDI during both the sensitization and effector phases (*table 3*). Neither of the RP 67580 treatment regimes had a significant effect on the sensitivity (EC50 value) of control or TDI-sensitized mice (*table 3*). In all

these experiments RP 68651 had no effect on the tracheal hyperreactivity observed in TDI-sensitized mice (*table 3*).

Effect of CGRP and $CGRP_{8-37}$ on tracheal hyperreactivity

The effect of CGRP on the TDI-induced tracheal hyperreactivity 24 hr after the challenge was measured. Administration of CGRP around the time of challenge (-12, -1 and +5 hr) had no significant effect on tracheal hyperreactivity induced by exposure to TDI (*figure 4*). The TDI-sensitized groups, treated with saline or CGRP, exhibited hyperreactivity to carbachol when compared with the relevant nonsensitized groups (Emax: nonsensitized-saline-treated 2150 ± 97 mg; TDI-sensitized-saline-treated 3027 ± 287 mg; nonsensitized-CGRP-treated 2214 ± 251 mg; TDI-sensitized-CGRP-treated 2995 ± 305 mg, mean ± sem, for n=7-8 mice/group, $P<0.005$). $CGRP_{8-37}$ (0.2 µmol/kg, i.v.) did not influence the TDI-induced tracheal hyperreactivity either (Emax: nonsensitized-saline-treated 2182 ± 86 mg; TDI-sensitized-saline-treated 2837 ± 177 mg; nonsensitized-$CGRP_{8-37}$-treated 1907 ± 101 mg; TDI-sensitized-$CGRP_{8-37}$-treated 2769 ± 312 mg; mean ± sem for n=6-12 mice/group, $P<0.05$).

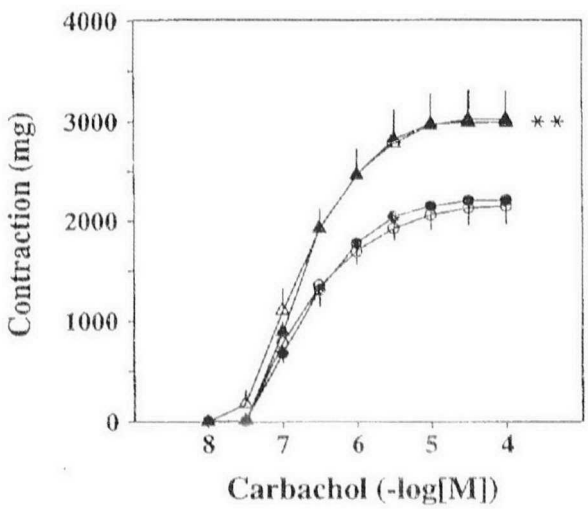

Figure 4 *Tracheal reactivity after CGRP treatment around the time of the challenge. Concentration-response curves to carbachol were measured in trachea of nonsensitized (circles) and TDI-sensitized (triangles) mice after CGRP (closed symbols) or vehicle (open symbols) treatment during the challenge (-12 hr, -1 hr and +5 hr). Results are expressed as mean ± sem for n=7-8 mice/group. Significant differences ($P<0.01$) between the nonsensitized and TDI-sensitized curves are denoted by (**).*

Table 4. Increase in ear swelling (x 10^{-2} mm) of TDI-sensitized and nonsensitized mice 24 hr after the challenge. The effect of capsaicin, RP 67580, CGRP and $CGRP_{8-37}$ treatment.

Treatment	Nonsensitized	TDI-sensitized
Vehicle	0.071 ± 0.011	0.260 ± 0.007**
Capsaicin	0.065 ± 0.002	0.200 ± 0.023**
RP 68651	0.077 ± 0.012	0.334 ± 0.038**
RP 67580	0.085 ± 0.008	0.389 ± 0.043**
Vehicle	0.062 ± 0.010	0.299 ± 0.048**
CGRP	0.063 ± 0.007	0.284 ± 0.027**
Vehicle	0.077 ± 0.031	0.238 ± 0.034*
$CGRP_{8-37}$	0.018 ± 0.008	0.311 ± 0.022**

Results are expressed as mean ± sem for n=4-6 mice/group. Significant differences ($P<0.05$ and $P<0.01$) between TDI-sensitized and nonsensitized mice are denoted by (*) and (**).

Cutaneous responses

In addition to tracheal reactivity, the development of cutaneous responses was also followed. TDI sensitization followed by topical challenge with TDI resulted in a marked and significant increase in ear swelling 24 hr after the challenge when compared with the nonsensitized mice (*table 4*). In contrast to the effects on tracheal hyperreactivity, none of the treatments (i.e. capsaicin-pretreatment, RP 67580 administration, CGRP and $CGRP_{8-37}$ treatment) had a significant effect on the TDI-induced ear swelling response (*table 4*).

DISCUSSION

In a previous study we have developed a model to study occupational asthma that is induced by repeated exposure to TDI (sensitization phase and a challenge phase) (274). In the present study we have demonstrated the important role for sensory neuropeptides, particularly the tachykinins, in the development of tracheal hyperreactivity associated with this model. Direct application of TDI induced a concentration-dependent relaxation of the mouse trachea *in vitro*. SP also induced a concentration-dependent relaxation of the mouse

trachea *in vitro*. Both these responses were completely inhibited by the selective NK_1 receptor antagonist RP 67580. Additionally, capsaicin pretreatment blocked the TDI-induced relaxation whereas the SP-induced relaxation remained equal. These results clearly demonstrate that TDI is capable of releasing a tachykinin in the murine trachea. It has been demonstrated that *in vitro* in the rat bladder (180) and in the guinea pig airway smooth muscle (176, 182) TDI induced a contraction through the activation of the efferent function of capsaicin-sensitive sensory nerves. In these latter studies it was also shown that tachykinin release from the peripheral endings resulted in mast cell degranulation and the subsequent release of bioactive mediators (176). Mapp and coworkers showed that TDI was able to contract or to relax human bronchial smooth muscle (60). They showed that capsaicin provoked the same responses in the human bronchi, suggesting that TDI and capsaicin act similarly in isolated human airways. From these studies it can be concluded that TDI can stimulate sensory nerves. However, a discrepancy exists between species in the effect sensory neuropeptides have on smooth muscle activity. In man, guinea pig and rat release of sensory neuropeptides resulted in contraction of the airway smooth muscle, whereas tachykinins produced a relaxation in the mouse airway smooth muscle (174).

We have developed a murine model to investigate TDI-induced asthma (274). This model exhibits some of the important features of clinical TDI-induced asthma. After skin sensitization with TDI (1%) followed by a intranasal challenge (1% TDI) nonspecific tracheal hyperreactivity to carbachol and serotonin was found in TDI-sensitized mice compared with nonsensitized mice (274). The results from the present study clearly show that TDI is able to release tachykinins from sensory nerves in the tracheal smooth muscle of the mouse. Moreover, we found that after capsaicin pretreatment TDI was no longer able to induce tracheal hyperreactivity. These results suggest an important role for sensory nerves and neuropeptides in the induction of tracheal hyperreactivity after exposure to TDI. Since capsaicin pretreatment, resulting in sensory neuropeptide depletion, was performed before sensitization, the difference phases (i.e. sensitization and effector phases) could not be examined separately. Therefore, we tested the effect of a selective NK_1 antagonist, RP 67580, during the sensitization and effector phases. RP 67580 was found to inhibit the TDI-induced tracheal hyperreactivity when administered before the challenge. However, the TDI-induced tracheal hyperreactivity was not suppressed when RP 67580 was administered during the sensitization phase. Takeda and coworkers showed in a model for nasal allergy in the guinea pig that sensory neuropeptide depletion before sensitization with TDI resulted in suppression of nasal allergy-like symptoms (294). Thompson and coworkers demonstrated that TDI-induced airway hyperresponsiveness in spontaneously

breathing guinea pigs was prevented by capsaicin pretreatment and the tachykinin receptor antagonist (D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11])SP (297). This latter model however, differed quite considerably from our mouse model because the responses were measured 2 hr after a 1 hr exposure to TDI and, in our opinion, in this latter model the irritant effect after TDI exposure and not the effect of repeated exposure to TDI was investigated.

While it is clear from this present study that the tachykinins are involved in TDI-induced tracheal hyperreactivity, it is not known which cells are the target for these mediators. A direct effect of tachykinins in the induction of tracheal hyperreactivity in smooth muscle is not likely because they have been shown to induce relaxation in the mouse airways. Interestingly, in our previous studies it has been proposed that T cells play an important role in the TDI-induced tracheal hyperreactivity (274). It is known that tachykinins can concentration-dependently enhance the proliferation of mitogen-stimulated T-lymphocytes (56, 234, 287). In addition, SP has been reported to activate macrophages (145), induce the production of cytokines (162) and facilitate the migration of macrophages, neutrophils, and T lymphocytes (187, 207). Finally, it has been demonstrated that SP activates mast cells of the respiratory system (114), resulting in the release of bioactive mediators. It is therefore possible that sensory neuropeptides indirectly modulate tracheal smooth muscle reactivity during the effector phase via one of these mechanisms. We have clearly shown that upon intranasal challenge with TDI tachykinins are released from the sensory nerve endings in the airways. We hypothesize that the tachykinins then stimulate antigen-specific T lymphocytes, macrophages, mast cells or a combination of these cell populations. The subsequent release of bioactive mediators (i.e. cytokines, histamine) could lead to the observed tracheal hyperreactivity in the mouse airways.

Several reports have suggested a proinflammatory activity for CGRP (45, 46, 92). However, in our model $CGRP_{8-37}$ was unable to inhibit the TDI-induced tracheal hyperreactivity and the cutaneous responses. Recently, CGRP has been shown to inhibit a cutaneous DTH reaction when administered before the induction (sensitization) phase (8). In light of these experiments it was thought that CGRP itself might suppress the TDI-induced tracheal hyperreactivity or cutaneous responses during the effector phase. However, CGRP did not influence the TDI-induced responses in either the trachea or the skin and we conclude that CGRP does not play a role in these reactions.

In contrast to the tracheal hyperreactivity, the cutaneous hypersensitivity responses induced by TDI were not influenced by sensory neuropeptides. Neither capsaicin, RP 67580, CGRP nor $CGRP_{8-37}$ treatment had any effect on the TDI-induced ear swelling response 24 hr after the challenge. These results are in agreement with a previous study

from our group (47). In this report it was demonstrated that DTH reactions in the skin were not affected by capsaicin pretreatment whereas tracheal hyperreactivity was abolished. Interestingly, in the same study leukocyte accumulation and mucosal exudation in the bronchoalveolar lavage fluid were markedly enhanced after the systemic depletion of sensory neuropeptides, leading to the possibility that it was not a difference between the airways and skin that was important but a difference between airway smooth muscle reactivity and inflammation (leukocyte accumulation and edema). In agreement, Thompson and coworkers also found a discrepancy between TDI-induced airway hyperresponsiveness and airway edema. Capsaicin pretreatment abolished the TDI-induced increase in airway hyperresponsiveness in contrast to both the neutrophil accumulation and the tracheal edema which were slightly increased after capsaicin pretreatment (297).

In summary, we have shown that sensory neuropeptides and particularly tachykinins are involved in the effector phase of TDI-induced tracheal hyperreactivity. The tachykinins do not act directly on the tracheal smooth muscle but their effects are probably mediated through the activation of other cells (i.e. T lymphocytes or mast cells). Clarification of the mechanism of action of the tachykinins will undoubtedly lead to a better understanding of the pathophysiological events prevalent to occupational asthma and may offer new targets for therapeutic intervention.

CHAPTER 5

Relationship between toluene diisocyanate-induced mast cell activation and tracheal hyperreactivity

G 02

Relatic nship between toluene diisocyanate-induced mast cell activation and tracheal hyperreactivity

Heleen Scheerens, Theresa L. Buckley, Thea Muis, Judith Scheerens, Johan Garssen, Henk Van Loveren, Frans P. Nijkamp.

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ABSTRACT

Toluene diisocyanate (TDI) is a low molecular weight compound which is a well known cause of occupational asthma. To investigate the mechanisms of TDI-induced occupational asthma we have recently developed a murine model. Skin sensitization followed by intranasal challenge with 1% TDI resulted in nonspecific *in vitro* tracheal hyperreactivity which was not associated with an increase in TDI-specific IgE antibodies. It was hypothesized that an IgE-independent, delayed-type hypersensitivity (DTH)-like mechanism could be involved in TDI-induced occupational asthma. In the present study, it was demonstrated that skin sensitization with 1% TDI caused marked mucosal mast cell activation that started before and lasted until 24 hr after the challenge. In further experiments, the role of TDI-induced mast cell activation in the development of tracheal hyperreactivity was investigated in two ways. First, mast cell deficient (W/W^v) mice were used. Interestingly, W/W^v mice exhibited TDI-induced tracheal hyperreactivity 24 hr after the challenge, indicating that mast cells are not mandatory for the induction of tracheal hyperreactivity. Second, the role of the mast cell mediators histamine and serotonin was investigated using specific receptor antagonists. Treatment with cimetidine (16 mg/kg, i.p.), a histamine H_2 receptor antagonist, around the time of challenge abrogated the TDI-induced tracheal hyperreactivity, demonstrating that histamine contributed to the TDI-induced tracheal hyperreactivity. In agreement, no tracheal hyperreactivity was observed in TDI-sensitized mice after treatment with ketanserin (16 mg/kg, i.p.), a serotonin $(5\text{-HT})_2$ receptor antagonist. However, ketanserin itself did lead to an increased tracheal reactivity in the nonsensitized mice. Taken together, these results illustrated that histamine, through activation of H_2 receptors, and serotonin, through activation of 5-HT_2 receptors, are probably involved in the development of TDI-induced tracheal hyperreactivity. Whether the mast cell is the source for these mediators in this reaction is unclear and requires further investigations.

INTRODUCTION

Mast cells have been implicated in the pathogenesis of a number of inflammatory diseases including asthma (90). Mast cells are reported to be both increased and activated in the airways of allergic asthmatic subjects (237). In allergic asthma, mast cells are thought to be activated through the FceRI receptor that binds IgE (88). The subsequent release of

mediators (histamine, serotonin, cytokines) contributes to the features of asthma (38). In addition, mast cells have also been shown to play an important role in occupational asthma induced by isocyanates, which are among one of the most serious causes of occupational asthma. Toluene diisocyanate (TDI)-induced occupational asthma is characterized by nonspecific hyperresponsiveness of the airways and an influx of inflammatory cells, such as T lymphocytes, eosinophils and neutrophils (34, 264). In subjects with TDI-induced occupational asthma, mast cell degranulation has been detected in the bronchial mucosa by electron microscopy (262). Additionally, it has been found that the number of mast cells in the airway mucosa was inversely correlated with the length of exposure to TDI before the onset of asthma symptoms (288). Moreover, mediators released from mast cells have also been shown to be increased in subjects with TDI-induced asthma. For example, neutrophil chemotactic activity, associated with mast cell or basophil activation, was increased in serum (272) and tumor necrosis factor–α (TNF–α), which is also released by mast cells, was increased in bronchoalveolar lavage (BAL) fluid of subjects with TDI-induced occupational asthma (169).

Although TDI-induced occupational asthma has been studied extensively, very little is known about the mechanisms of action of TDI. Recently, we developed a murine model to investigate TDI-induced asthma (274). Skin sensitization on day 0 and day 1 followed by intranasal challenge on day 8 with 1% TDI resulted in the mimicking of most of the characteristics found in TDI-induced occupational asthma: no increase in TDI-specific IgE antibodies, tracheal hyperreactivity 24 hr after the challenge, and increased myeloperoxidase activity in the lung tissue and airway lumen which is indicative of neutrophil activation (274). We hypothesized that TDI-induced asthma was regulated via a delayed-type hypersensitivity (DTH)-like reaction, since no IgE was found and T lymphocytes have been suggested to play an important role (274). DTH reactions are T cell-dependent immune responses and have been investigated using the skin as the site of reaction. Moreover, in our departments several murine models have been developed to investigate DTH reactions in the lung using dinitrofluorobenzene (DNFB) and picryl chloride as sensitizing haptens (47, 95). Skin sensitization with picryl chloride followed by intranasal challenge with 0.6% picryl sulphonic acid, a water soluble form of picryl chloride, resulted in an accumulation of mononuclear inflammatory cells around bronchioli and blood vessels (95). These lung DTH responses were severely reduced in mast cell deficient *W/W*^{v} mice suggesting an important role for mast cells (96).

In the present study, the effect of TDI-sensitization and challenge on the mast cell in the mouse was investigated. Subsequently, the role of the mast cell in the induction of TDI-

induced tracheal hyperreactivity was studied, using mast cell deficient (W/W^v) mice and specific histamine and 5-HT receptor antagonists.

MATERIALS AND METHODS

Animals

Male BALB/c mice (6-8 weeks of age) were supplied either by the Central Animal Laboratory, Utrecht, The Netherlands or by the National Institute of Public Health and the Environment, Bilthoven, The Netherlands. Male mast cell-deficient mice ($WBB6F_1$-W/W^v) and their mast cell-sufficient congenic ($WBB6F_1$-+/+) mice (6-8 weeks of age) were purchased from Jackson Laboratories, Bar Harbor, ME, U.S.A. They were housed in groups not exceeding 6 per cage and maintained under standard conditions. All experiments were assessed by the animal ethics committee at Utrecht University and the National Institute of Public Health and the Environment.

Sensitization procedure

Mice were sensitized twice daily on day 0 and day 1 either with 1% TDI (sensitized group) dissolved in acetone:olive oil (4:1) or with vehicle control (nonsensitized group) which was applied epicutaneously to the shaved abdomen and thorax (100 µl) and four paws (100 µl) under light anaesthesia (sodium pentobarbitone; 50 µl; 30 mg/kg i.p.).

Challenge procedure

TDI-sensitized and nonsensitized groups were challenged intranasally with 1% TDI dissolved in ethyl acetate:olive oil (1:4) on day 8. Twenty µl of the TDI solution was applied intranasally under light anaesthesia (sodium pentobarbitone; 50 µl; 30 mg/kg i.p.). Furthermore, mice were also challenged on the ears; TDI (20 µl; 0.5%; dissolved in acetone) or vehicle control (20 µl) were applied topically to both sides of the ears.

Administration of 5-HT and histamine receptor antagonists

TDI-sensitized and nonsensitized mice were treated with ketanserin, a serotonin $(5\text{-HT})_2$ receptor antagonist, and cimetidine, a H_2 receptor antagonist, around the time of challenge to investigate the role of these mast cell mediators. Ketanserin and cimetidine were administered i.p. 18 hr and 5 minutes before the challenge and 2 hr and 8 hr after the challenge (total dose 16 mg/kg).

Histological analysis

At 2, 24 and 48 hr after the challenge trachea and lungs were removed from mice after lethal anaesthesia with 50 µl of a cocktail consisting of 7 ml of 50 mg/ml ketalar, 3 ml 2% rompun, and 1 ml of 1 mg/ml atropine, injected intramuscularly (Nembutal causes vasodilatation, which negatively influences the histology preparations). Before removing the lungs the mice were perfused with 5 ml phosphate buffered saline (PBS, 37°C) in the right heart ventricle. The lungs and trachea were filled intratracheally with a fixative (0.8%

formalin, 4% acetic acid) using a ligature around the trachea. The unfolded lungs and trachea were fixed for at least 24 hr in the fixative, dehydrated, and embedded in paraplast. Four µm thick sections were stained with periodic acid Schiff reagent in combination with toluidine blue. The difference in total number of mast cells was determined in the lungs of TDI-sensitized and nonsensitized mice.

Measurement of mouse mast cell protease-1 (MMCP-1)

Levels of MMCP-1 were measured before the challenge and 30 minutes, 4 hr and 24 hr after the challenge in the serum and lung tissue of TDI-sensitized and nonsensitized mice using a commercially available ELISA assay (Moredun Scientific Ltd., Scotland). After perfusing the mice with 5 ml PBS (37°C) in the right heart ventricle the lungs were isolated and homogenated in 1.5 M KCl at 4°C. The lung homogenates were centrifuged for 10 minutes at 10,000 g and the supernatants were used in the MMCP-1 assay. Nunc-immunoplates (Gibco BRL, Life Technologies, Breda, The Netherlands) were coated with 50 µl sheep anti-MMCP-1 capture antibody (1 µg/ml in 0.1 M carbonate buffer, pH 9.6) and incubated in a humid chamber at 4°C for 24 hr. Plates were washed 5 times with PBS/Tween 20 (0.05% v/v) and to block the non-bound places, plates were incubated for 30 minutes at 4°C with 50 µl 1% BSA in PBS/Tween 20. After washing the plates 5 times with PBS/Tween 20, 50 µl sample (1/5 diluted) or MMCP-1 standard (20 ng/ml) was added. Plates were incubated overnight at 4°C and washed again 5 times with PBS/Tween 20. Subsequently, 50 µl 1/300 diluted rabbit anti-MMCP-1-HRPO conjugate was added. After a 2 hr incubation at 4°C, the plates were washed again 5 times with PBS/Tween 20 and incubated for 30 minutes at room temperature with 50 µl o-phenylenediamine dihydrochloride (2.2 mM in 0.1 M citric/phosphate buffer, pH 5.0). The reaction was stopped by adding 25 µl 2.5 M H_2SO_4 and the absorption was measured at 492 nm using a microplate reader.

Isometric measurement of tracheal reactivity

Tracheal reactivity was measured using the method of Garssen et al. (97). Mice were killed by an overdose of sodium pentobarbitone (0.3 ml; 60 mg/kg i.p.). The trachea, which was resected *in toto*, was carefully cleaned of connective tissue using a binocular microscope. A 9 ring piece of the trachea (taken from just below the larynx) was then transferred to a 10 ml organ bath containing a modified oxygenated Krebs solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCL_2 6H_2O$, 0.5 mM $MgCl_2 6H_2O$, 25.0 mM $NaHCO_3$, 1.0 mM $NaHPO_4 H_2O$, 11.1 mM glucose). The trachea was directly slipped onto 2 supports of an organ bath one of which was coupled to the organ bath and the other to an isometric transducer. The solution was aerated (95%: 5%; O_2: CO_2) at a constant temperature (37°C). Isometric measurements were made using a force displacement transducer (Harvard Bioscience, Boston, MA) and a 2 channel recorder (Servogar type SE-120) and were expressed as change in mg force. Optimal pre-load for the mouse trachea was determined to be 1000 mg. The trachea was allowed to equilibrate for at least 1 hr before drug effects were elicited. During the equilibrium phase the fluid in the bath was changed every 15 minutes. To assess reactivity concentration-response curves to carbachol (10^{-8}-10^{-4} M) were determined 24 hr after challenge.

Chemicals

Toluene diisocyanate, olive oil, carbachol, o-phenylenediamine dihydrochloride were purchased from Sigma Chemical Co. St. Louis, U.S.A. Tween 20 and ketanserin were purchased from Janssen Pharmaceutical, Belgium. Cimetidine was purchased from SmithKline and Beecham Pharmaceuticals, U.S.A. Sodium pentobarbitone was purchased from Sanofi, Maassluis, The Netherlands.

Statistics

All experiments were designed as completely randomized multifactorials with 6-14 mice per group. EC50 and Emax values for the carbachol-induced tracheal contractions of each experimental animal were calculated separately by nonlinear least-squares regression analysis (simplex minimalization) of the measured contractions vs. carbachol concentration using the sigmoid concentration-response relationship and including a threshold value. The data were analysed by two-way Analysis of Variance followed by a post-hoc comparison between groups. In the figures and tables group means ± sem are given and a difference was considered significant when $P<0.05$. All data manipulation, non-linear fittings, Analyses of Variance and post-hoc comparisons were carried out with a commercially available statistical package (SYSTAT, version 5.03; Wilkinson L. SYSTAT: The system for statistics. Evanston, IL: SYSTAT, Inc., 1990. Statistics).

RESULTS

Effect of TDI treatment on the mast cell

Light microscopy and a toluidine blue staining were used to detect the presence of mast cells in the lung tissue and in the ear skin. In both TDI-sensitized and nonsensitized mice mast cells were easily detected. However, TDI-sensitization did not cause an increase in the number of mast cells in the lung and in the ear at 2, 24 and 48 hr after the challenge when compared with nonsensitized mice. In addition, light microscopy did not reveal degranulation and activation of the mast cells. Therefore, mouse mast cell protease - (MMCP-1, a specific marker for mouse mucosal mast cells) was measured in the lung tissue and serum using a standard MMCP-1 ELISA assay. *Figure 1A* clearly demonstrates that at 30 minutes, 4 hr and 24 hr after, and even before, the challenge the MMCP-1 levels were increased in the lungs of TDI-sensitized mice when compared with nonsensitized mice. The MMCP-1 levels in the lung tissue are a measurement for accumulation and activation of the mucosal mast cells. Additionally, the MMCP-1 levels in the serum were measured to ascertain the activation state of the systemic mucosal mast cell. Evidently, the MMCP-1 levels in the serum were also significantly increased before and at all time points

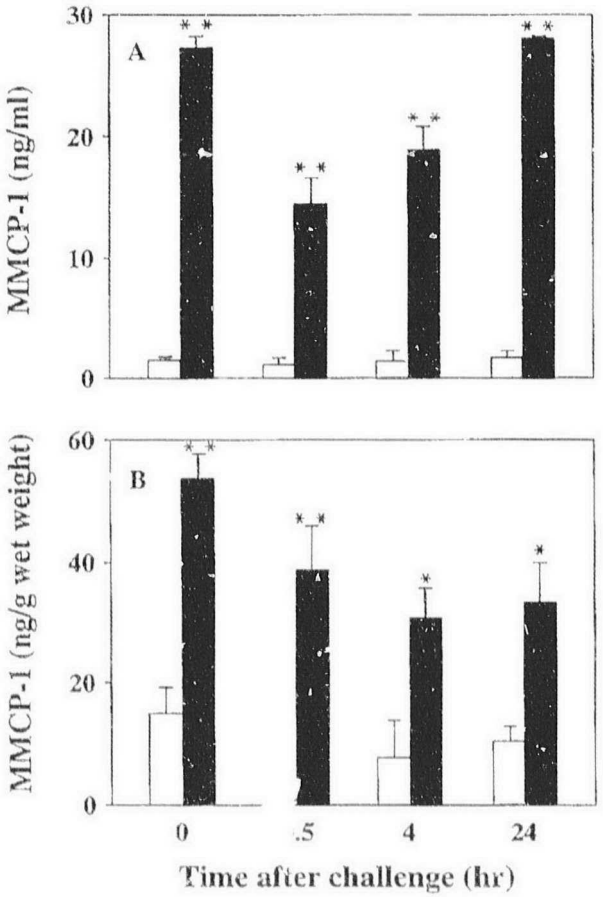

Figure 1 Mouse mucosal mast cell protease-1 (MMCP-1) levels in (A) lung tissue and (B) serum. MMCP-1 levels were measured in TDI-sensitized (closed bars) and nonsensitized (open bars) mice at t = 0, 0.5, 4 and 24 hr after the challenge. Results are expressed as mean ± sem for n=6 mice/group. Significant differences ($P<0.05$ and $P<0.01$) between TDI-sensitized and nonsensitized mice are denoted by (*) and (**) respectively.

after the challenge in TDI-sensitized mice when compared with the nonsensitized mice (*figure 1B*).

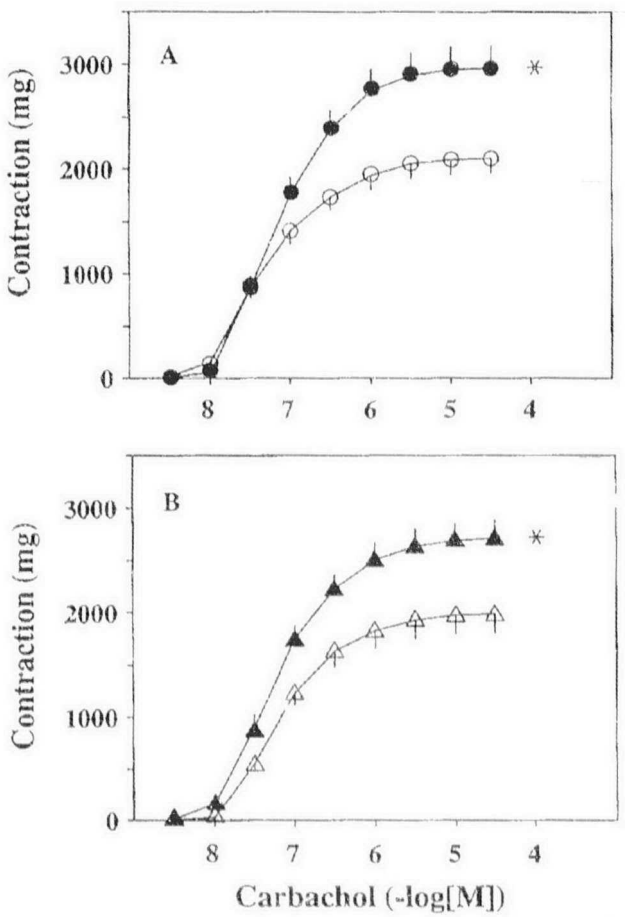

Figure 2 Tracheal reactivity to carbachol 24 hr after the challenge in (A) mast cell sufficient (+/+) mice and (B) mast cell deficient (W/W^v) mice. Concentration-response curves to carbachol were measured in the trachea of TDI-sensitized (closed symbols) and nonsensitized (open symbols) mice 24 hr after the challenge. Results are expressed as mean ± sem [illegible] =14-15 mice/group. Significant differences between TDI-sensitized and nonsensitized mice ($P<0.05$) are denoted by (*).

Effect of TDI treatment in mast cell deficient mice.

To investigate the role of the mast cell in the induction of TDI-induced tracheal hyperreactivity we tested our model in mast cell-deficient (W/W^v) mice. First, the congenic mast cell-sufficient (+/+) mice were sensitized and challenged with TDI. *Figure 2A* shows

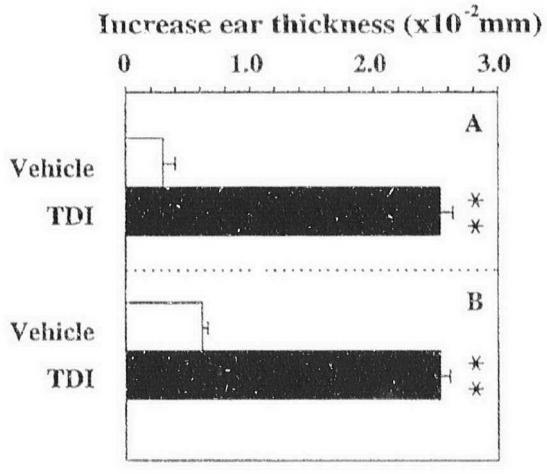

Figure 3 Ear swelling response 24 hr after the challenge in (A) mast cell sufficient (+/+) and (B) mast cell deficient (W/W^v) mice. TDI-sensitized (closed bars) and nonsensitized (open bars) mice were topically challenged on both ears; one ear with 0.5% TDI and the other with vehicle. The ear swelling was measured 24 hr after the challenge using a micrometer and the difference in ear thickness (x 10^{-2} mm) between the two ears is expressed as mean ± sem for n=5 mice/group. Significant differences between bars ($P<0.01$) are denoted by (*).

that TDI induced tracheal hyperreactivity to carbachol in the (+/+) mice 24 hr after the challenge (Emax: nonsensitized (+/+) 2134 ± 146 mg; TDI-sensitized (+/+) 2965 ± 215 mg; mean ± sem for n=14-15 mice/group, $P<0.01$). Surprisingly, the TDI-sensitized and challenged mast cell-deficient mice W/W^v mice were also significantly more reactive to carbachol when compared with the nonsensitized W/W^v mice (Emax: nonsensitized(W/W^v) 1984 ± 181 mg; TDI-sensitized (W/W^v) 2713 ± 172 mg; mean ± sem for n=14 mice/group, $P<0.05$). In both W/W^v and +/+ mice no difference in EC50 values were found between TDI-sensitized and nonsensitized mice (EC50: nonsensitized (+/+) 0.210 ± 0.023 x 10^{-6} M; TDI-sensitized (+/+) 0.164 ± 0.019 x 10^{-6} M; nonsensitized (W/W^v) 0.148 ± 0.020 x 10^{-6} M; TDI-sensitized (W/W^v) 0.194 ± 0.022 x 10^{-6} M; mean ± sem for n=14-15 mice/group).

In addition to pulmonary responses, the TDI-induced cutaneous responses were measured. In agreement with the tracheal hyperreactivity, the TDI-induced cutaneous ear swelling response was not abolished in mast cell deficient mice. *Figure 3* demonstrates that TDI-sensitization and challenge resulted in an increased ear swelling 24 hr after the challenge in both the mast cell-sufficient (+/+) and the mast cell deficient (W/W^v) mice.

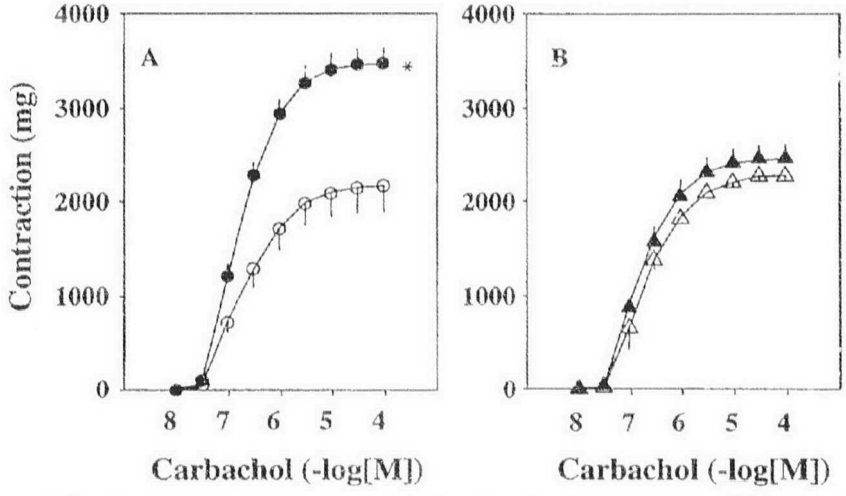

Figure 4 Tracheal reactivity to carbachol 24 hr after the challenge in (A) PBS-treated and (B) cimetidine-treated mice. Concentration-response curves to carbachol were measured in the trachea of TDI-sensitized (closed symbols) and nonsensitized (open symbols) mice 24 hr after the challenge. Results are expressed as mean ± sem for n=3-10 mice/group. Significant differences between TDI-sensitized and nonsensitized mice (P<0.05) are denoted by (*).

Effect of 5-HT and histamine receptor antagonists on TDI-induced responses.

The role of the mast cell mediators histamine and serotonin was investigated by treatment with selective receptor antagonists. First, administration of cimetidine (16 mg/kg, i.p.), a selective H_2 receptor antagonist, around the time of challenge caused a significant inhibition of the TDI-induced tracheal hyperreactivity (*figure 4*. Emax: nonsensitized 2161 ± 280 mg; TDI-sensitized 3470 ± 174 mg; nonsensitized + cimetidine 2282 ± 50 mg; TDI-sensitized + cimetidine 2463 ± 156 mg, mean ± sem for n=3-10, P<0.05). To exclude eventual histamine H_1 receptor activity of cimetidine, the selective histamine H_1 receptor antagonist mepyramine was tested on TDI-induced tracheal hyperreactivity using a similar regime. Mepyramine (16 mg/kg, i.p.) did not inhibit the TDI-induced tracheal hyperreactivity when it was administered around the time of challenge (data not shown).

Second, the 5-HT_2 receptor antagonist, ketanserin (16 mg/kg, i.p.) was administered to nonsensitized and TDI-sensitized mice around the time of challenge. There was no difference between the nonsensitized ketanserin-treated and TDI-sensitized ketanserin-treated group, suggesting that ketanserin blocked the TDI-induced hyperreactivity to

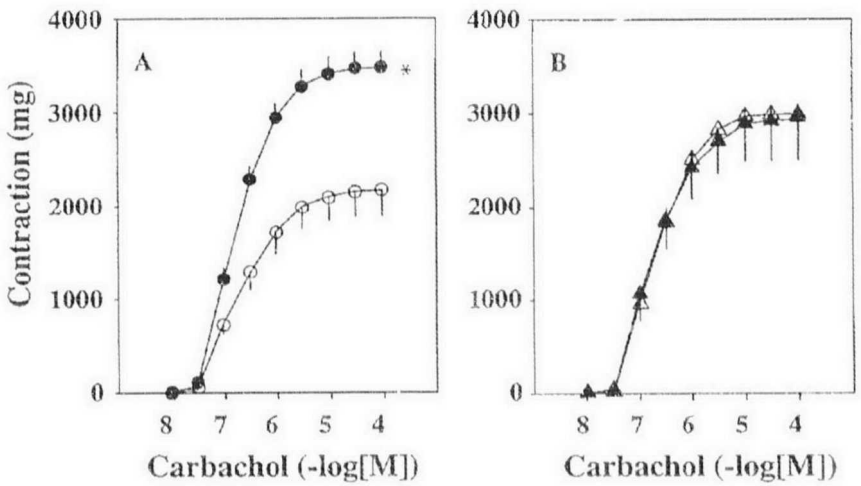

***Figure** 5 Tracheal reactivity to carbachol 24 hr after the challenge in (A) PBS-treated and (B) ketanserin-treated mice. Concentration-response curves to carbachol were measured in the trachea of TDI-sensitized (closed symbols) and nonsensitized (open symbols) mice 24 hr after the challenge. Results are expressed as mean ± sem for n=3-10 mice/group. Significant differences between TDI-sensitized and nonsensitized mice (P<0.05) are denoted by (*).*

carbachol (*figure 5*). However, the nonsensitized ketanserin-treated mice reacted significantly stronger to carbachol when compared with the nonsensitized mice (Emax: nonsensitized 2161 ± 280 mg; TDI-sensitized 3470 ± 174 mg; nonsensitized + ketanserin 2987 ± 496 mg; TDI-sensitized + ketanserin 2931 ± 191 mg, for n=3-9 mice/group, P<0.05). In contrast to the effect on the pulmonary response, neither cimetidine nor ketanserin treatment around the time of challenge inhibited the TDI-induced ear swelling 24 hr after the challenge (*table 1*).

DISCUSSION

In the present study the effect of TDI-sensitization and challenge on mast cells in the mouse was investigated. The increases in MMCP-1 in serum and lung tissue of TDI-sensitized mice have clearly demonstrated that TDI was able to induce mast cell activation in the mouse after skin sensitization. This finding is in agreement with pathological data

Table 1. Effect of cimetidine and ketanserin of the TDI-induced ear swelling 24 hr after the challenge.

Sensitization	Treatment	ear swelling (10^{-2} mm)
Control	PBS	0.070 ± 0.019
TDI	PBS	0.275 ± 0.039*
Control	Cimetidine	0.032 ± 0.009
TDI	Cimetidine	0.225 ± 0.025*
Control	Ketanserin	0.041 ± 0.016
TDI	Ketanserin	0.225 ± 0.094*

Results are expressed as mean ± sem for n=5-6 mice/group. Significant differences between nonsensitized and TDI sensitized mice are denoted by *P<0.05.

from subjects with TDI-induced occupational asthma. Fabbri and coworkers have demonstrated that the number of mast cells in the epithelium of bronchial biopsies of subjects with TDI-induced asthma was significantly increased (262, 288). Moreover, electron microscopy studies showed marked degranulation of mast cells in the bronchial epithelium of subjects with TDI-induced asthma (262). Furthermore, it has been demonstrated that inhalation challenge with subirritant levels of TDI caused an increase in neutrophil chemotactic activity (NCA), which is associated with mast cell or basophil activation (272). The increase in NCA was correlated with significant decreases in FEV_1 (forced expiratory volume in first second), suggesting that mast cell or basophil activation was associated with TDI-induced asthmatic reactions (272). In addition, in several animal models, exposure to TDI (inhalation or intradermal) resulted in increased numbers and activation of mast cells (61, 128, 183). Taken together, these data confirm that TDI activates mast cells in human as well as in mice, rats and guinea pigs. However, the function of TDI-induced mast cell activation in the development of TDI-induced occupational asthma is not well understood.

Previously, we have developed a model to investigate TDI-induced pulmonary changes (274). In this model, in which DTH-like responses rather than IgE are involved, the role of the mast cell in TDI-induced tracheal hyperreactivity was investigated. Skin sensitization on day 0 and day 1 followed by intranasal challenge on day 8 with 1% TDI caused a significant *in vitro* tracheal hyperreactivity in mast cell deficient *W/W*^{v} mice 24 hr after the

challenge. These data suggest that mast cells are not required for the development of TDI-induced tracheal hyperreactivity. However, recent studies have raised considerable doubt about the usefulness of mast cell deficient W/W^v mice. First, it has been reported that W/W^v mice still contain 1% of their mast cells and this might have been sufficient to elicit pulmonary DTH-like responses (9, 248). Second, in agreement with our results, Askenase and coworkers found no abolishment of picryl chloride- and oxazolone-induced DTH reaction in the skin of W/W^v mice (10, 102). They demonstrated that the cutaneous DTH responses in W/W^v mice could be inhibited by depletion of platelets and suggested that in W/W^v mice platelets act as an additional source of serotonin, mediating DTH reactions (102). Third, it has been shown that repeated application of the contact sensitizing agent TNCB to the ears of W/W^v mice resulted in increased mast cell numbers in the skin which was not induced in another mast cell deficient mouse strain, Sl/Sl^d mice (143). This induction could have also occured after TDI-sensitization and challenge of W/W^v mice and in further experiments this possibility will be examined.

Due to the reservations outlined above, the role for mast cell mediators was examined in the development of tracheal hyperreactivity. The role for histamine and serotonin was determined using specific histamine and 5-HT receptor antagonists. Interestingly, administration of cimetidine, a H_2 receptor antagonist (16 mg/kg, i.p.), around the time of challenge completely abrogated the TDI-induced tracheal hyperreactivity 24 hr after the challenge, whereas treatment with the histamine H_1 receptor antagonist mepyramine (16 mg/kg, i.p.) had no effect. These results indicated that histamine is involved in DTH-like TDI-induced tracheal hyperreactivity through activation of H_2 receptors. Additional sources of histamine are basophils and neurons. For example, it has been demonstrated that cutaneous DTH reactions elicited by dinitrochlorobenzene were associated with an increase in serum histamine concentration, which was suggested to be dependent on the number of basophils, and not mast cells, infiltrating the lesion site (293). It is therefore possible that mast cells are not the exclusive source of histamine in the responses outlined in this chapter.

Furthermore, the H_2 receptor has been detected on mast cells, T lymphocytes and probably on eosinophils (30, 249, 312). The H_2 receptors on these immune cells are able to modulate the secretion of bioactive mediators. For example, it has been demonstrated that histamine inhibits the tumor necrosis factor–α release by mast cells through H_2 and H_3 receptors (30). The role of histamine and H_2 receptors has been investigated in cutaneous DTH reactions. Several studies reported no effect of cimetidine on cutaneous DTH reactions when it was administered around the time of challenge (111, 160, 229, 266). In

agreement with these findings, the TDI-induced cutaneous response was unaffected in the present study. In contrast, it has been demonstrated that treatment with cimetidine before and during the sensitization phase (6 consecutive weeks) caused an augmented cutaneous DTH response (13), although considerable higher dosages were used when compared with our study. In conclusion, the cimetidine-induced inhibition of DTH-like TDI-induced tracheal hyperreactivity is an interesting and novel finding and suggests that histamine is an important mediator in TDI-induced pulmonary changes.

The role of serotonin in the development of cutaneous DTH reactions has been extensively studied. It has been established that cutaneous DTH responses in the mouse depend on the release of serotonin from mast cells during the early phase of DTH reactions (4, 307). Serotonin is capable of activating 5-HT_2 receptors on blood vessels, resulting in increased vascular permeability, thus promoting the influx of DTH effector T cells (11). Additionally, it has been demonstrated that in picryl chloride-induced ear swelling the inhibiting effect of ketanserin was due to blocking of 5-HT_2 receptors on the DTH effector T cells itself (4). Moreover, the picryl chloride-induced mononuclear cell influx into the airways has been demonstrated to be markedly reduced after treatment with ketanserin during the challenge phase (96). Likewise, in this present study, there was no difference in tracheal reactivity between the ketanserin-treated nonsensitized mice and the ketanserin-treated TDI-sensitized mice.

In conclusion, the results of this study clearly demonstrate that TDI-sensitization leads to a marked activation of mucosal mast cells in the mouse. Furthermore, the TDI-induced tracheal hyperreactivity 24 hr after the challenge could be inhibited by treatment with the H_2 receptor antagonist cimetidine and probably also by the 5-HT_2 receptor antagonist ketanserin. The conclusion that histamine was involved during the effector phase of TDI-induced DTH-like pulmonary responses is innovative and reflects a new possible mechanism in TDI-induced occupational asthma.

CHAPTER 6

The role of $CD4^+$ and $CD8^+$ T-lymphocytes in toluene diisocyanate-induced tracheal hyperreactivity

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The role of $CD4^+$ and $CD8^+$ T-lymphocytes in toluene diisocyanate-induced tracheal hyperreactivity

Heleen Scheerens, Theresa L. Buckley, Thea Muis, Ellen M. Davidse, Frans P. Nijkamp, Henk Van Loveren.

ABSTRACT

Toluene diisocyanate (TDI) is a low molecular weight compound which is a well known cause of occupational asthma. Previously, we published a murine model to investigate TDI-induced occupational asthma. Skin sensitization on day 0 and day 1, followed by intranasal challenge with 1% TDI on day 8 resulted in a nonspecific *in vitro* tracheal hyperreactivity in the mouse airways, which was not associated with an increase in TDI-specific IgE antibodies. We hypothesized that an IgE-independent, delayed-type hypersensitivity (DTH)-like reaction could be involved in the development of TDI-induced asthma. In the present study, we investigated the role of T lymphocytes in the induction of TDI-induced tracheal hyperreactivity. First, the T cell dependency of the TDI-induced tracheal hyperreactivity was examined using athymic nude mice. In contrast to normal BALB/c mice no tracheal hyperreactivity was found in TDI-sensitized athymic nude mice. Second, adoptive transfer of lymphoid cells (5 x 10^6 cells/mouse) isolated from TDI-sensitized donor mice to naive recipients induced tracheal hyperreactivity 24 hr after the challenge when compared with the appropriate controls. Third, *in vivo* depletion of $CD4^+$ or $CD8^+$ T lymphocytes, by intraperitoneal injection of 100 mg monoclonal antibodies against CD4 or CD8 after the sensitization phase (day 3, 4 and 5), was performed. Depletion of $CD4^+$ or $CD8^+$ T lymphocytes markedly affected the TDI-induced tracheal hyperreactivity 24 hr after the challenge. This influence was most pronounced after depletion of $CD8^+$ T lymphocytes, which abolished this response. In conclusion, T lymphocytes play an important role in the DTH-like reaction elicited by TDI. Our results suggest that both $CD4^+$ and $CD8^+$ T cells act as DTH effector T cells in this reaction.

INTRODUCTION

Toluene diisocyanate (TDI) is a low molecular weight compound which is extensively used in various industries. Approximately 5-10% of the subjects exposed to TDI develop occupational asthma, characterized by a nonspecific airway hyperresponsiveness and an inflammation of the airways (238, 262, 288). Another important feature of TDI-induced occupational asthma is the lack of TDI-specific IgE antibodies in the serum of 80% of the subjects (21, 141). Recently, we have developed a murine model to investigate IgE-independent TDI-induced occupational asthma (274). This model was based on previous studies in our laboratories in which pulmonary delayed-type hypersensitivity (DTH)-like

reactions were induced by the low molecular weight haptens dinitrofluorobenzene (DNFB) and picryl chloride (47, 98). It was demonstrated that picryl chloride and DNFB induced tracheal hyperreactivity 24-48 hr after intranasal challenge which was associated with vascular leakage and inflammation of the airways (47, 98). The picryl chloride-induced tracheal hyperreactivity and inflammatory reactions in the lung were demonstrated to be T cell-mediated responses (96). In comparison, we have demonstrated in a previous study that skin sensitization on day 0 and day 1 twice daily with 1% TDI followed by intranasal challenge on day 8 (1% TDI) caused nonspecific *in vitro* tracheal hyperreactivity 24 hr after the challenge (274). Furthermore, the TDI-induced tracheal hyperreactivity could be transferred by lymphoid cells isolated from TDI-sensitized donor mice to naive recipients (274). These results indicated that lymphocytes could play an important role in TDI-induced tracheal hyperreactivity and that our initial results in this model were consistent with a DTH-like reaction.

DTH reactions can be elicited by small haptens which upon first contact (sensitization) bind to normal body proteins. Within 1 to 2 days after the sensitization DTH-initiating lymphocytes are activated to produce antigen-specific lymphocyte factors (150). These antigen-specific lymphocyte factors bind to mast cells (and possibly other cells (252)) in the circulation and upon local second antigen contact (challenge) the antigen binds to the factors on the mast cell (198). The subsequent release of mediators from the mast cell, such as serotonin, histamine and leukotrienes, attract circulating antigen-specific DTH effector T cells. These DTH effector T cells can recognize antigen in the context of major histocompatibility class II on antigen presenting cells (308). This event leads to the production of cytokines which causes the influx of other leukocytes and the consequent DTH response (308). It is generally thought that the DTH effector T cells are of the $CD4^+$ Th1 subset. However, recent studies on contact hypersensitivity (CHS) reactions induced by DNFB in the skin have shown that $CD8^+$ T lymphocytes could play an additional role in the induction of CHS (107).

In the present study, we investigated the role of T lymphocytes in the induction of TDI-induced tracheal hyperreactivity, using athymic nude BALB/c mice, adoptive transfer studies and *in vivo* depletion of specific T cell subsets ($CD4^+$ and $CD8^+$).

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MATERIALS AND METHODS

Animal

Male [illegible]B/c mice (6-8 weeks of age) were supplied either by the Central Animal Laboratory, Utrecht, The Netherlands or by the National Institute of Public Health and the Environment, Bilthoven, The Netherlands. Male athymic BALB/c-nu/nu mice were supplied by Harlan Netherlands B.V., Zeist, The Netherlands. They were housed in groups not exceeding 6 per cage and maintained under standard conditions. All experiments were assessed by the animal ethics committee at Utrecht University and the National Institute of Public Health and the Environment.

Sensitization procedure

Mice were sensitized twice daily on day 0 and day 1 either with 1% TDI (sensitized group) dissolved in acetone:olive oil (4:1) or with vehicle control (nonsensitized group) which was applied epicutaneously to the shaved abdomen and thorax (100 µl) and four paws (100 µl) under light anaesthesia (sodium pentobarbitone; 50 µl; 30 mg/kg i.p.).

Challenge procedure

TDI-sensitized and nonsensitized groups were challenged intranasally with 1% TDI dissolved in ethyl acetate:olive oil (1:4) on day 8. Twenty µl of the TDI solution was applied intranasally under light anaesthesia (sodium pentobarbitone; 50 µl; 30 mg/kg i.p.). Furthermore, on day 8 mice were also challenged on the ears; TDI (20 µl; 0.5%; dissolved in acetone) or vehicle control (20 µl) were applied topically to both sides of the ears.

Preparation of monoclonal antibodies

The following monoclonal antibodies generated in rats against murine antigens were used: YTS169 (rIgG2) against CD4 and YTS191 (rIgG2) against CD8. The hybridoma cells were cultured at 37°C in RPMI 1640 with 10% heat inactivated FCS and 0.05% gentamycin and grown to a maximal density. The rat monoclonal antibodies were purified from the supernatants of the hybridomas with the use of a Protein G-Sepharose affinity column (Pharmacia LKB, Uppsala, Sweden). The protein content was quantified by absorbance measurement at 280 nm.

Adoptive transfer of lymphoid cells

The inguinal lymph nodes and spleen from TDI-sensitized and nonsensitized mice were collected on day 6 after sensitization. They were pooled and cell suspensions were made by gently pressing the lymph nodes and spleen through a stainless steel screen. After washing the cell suspensions three times with cold phosphate buffered saline (PBS) total cell counts were performed. Five x 10^7 lymphoid cells in 100 µl PBS were transferred into the retroorbital plexus of normal recipient mice under sodium pentobarbitone anaesthesia (50 µl; 30 mg/kg i.p.). Twenty four hr later these recipients were challenged intranasally with 1% TDI and topically with 0.5% TDI, as described in the challenge procedure. Twenty four hr after this challenge tracheal reactivity to carbachol and cutaneous responses were measured.

In vivo depletion of $CD4^+$ or $CD8^+$ T lymphocytes

After TDI-sensitization on day 0 and day 1 mice were injected i.p. with 100 µg anti-CD4 antibody, anti-CD8 antibody or rIgG (control) on day 3, 4, 5 and 6; the nonsensitized mice were treated i.p. with 100 µg rIgG. On day 8 TDI-sensitized and nonsensitized mice were challenged intranasally and topically as described in the challenge procedure. Twenty four hr after the challenge tracheal reactivity to carbachol and cutaneous responses were measured. To determine the degree of depletion blood samples were taken by orbital puncture and lymph nodes and spleen were isolated for FACS analysis.

Characterization of cell subtypes in blood and lymphoid tissues

Blood samples were anticoagulated by mixing with acid citrate-glucose. An equal volume of hespan was added to the anticoagulated blood, mixed, and centrifuged for 15 minutes at 100 g to sediment erythrocytes. The remaining erythrocytes were lysed by an isotonic ammonium chloride solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.4) for 30 seconds at 37°C. The resultant leukocyte pellet was washed with PBS. Additionally, lymphoid cells isolated from the inguinal lymph nodes and spleen were washed twice with PBS. Hereafter, the leukocytes from the lymphoid tissue and the blood were incubated with 10% mouse serum for T cell labelling or with 5% rat serum for CD4, CD8 or B cell labelling. Subsequently, the cells were incubated with the FITC-labelled monoclonal antibodies Thy1.2, L3/T4, Ly-2 and Ly5 to determine the percentage of T cells, $CD4^+$ T cells, $CD8^+$ T cells and B cells, respectively. Finally, they were measured by flow cytometry under identical settings for all cell types (FACScan, Becton-Dickinson).

Isometric measurement of tracheal reactivity

Tracheal reactivity was measured using the method of Garssen et al. (97). Mice were killed by an overdose of sodium pentobarbitone (0.3 ml; 60 mg/kg i.p.). The trachea, which was resected *in toto*, was carefully cleared of connective tissue using a binocular microscope. A 9 ring piece of the trachea (taken from just below the larynx) was then transferred to a 10 ml organ bath containing a modified oxygenated Krebs solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCL_2 6H_2O$, 0.5 mM $MgCl_2 6H_2O$, 25.0 mM $NaHCO_3$, 1.0 mM $NaHPO_4 H_2O$, 11.1 mM glucose). The trachea was directly slipped onto 2 supports of an organ bath one of which was coupled to the organ bath and the other to an isometric transducer. The solution was aerated (95%: 5%; O_2: CO_2) at a constant temperature (37°C). Isometric measurements were made using a force displacement transducer (Harvard Bioscience, Boston, MA) and a 2 channel recorder (Servogar type SE-120) and were expressed as changes in mg force. Optimal pre-load for the mouse trachea was determined to be 1000 mg. The trachea was allowed to equilibrate for at least 1 hr before drug effects were elicited. During the equilibrium phase the fluid in the bath was changed every 15 minutes. To assess reactivity concentration-response curves to carbachol (10^{-8}-10^{-4} M) were determined 24 hr after challenge.

Measurement of cutaneous reactions

An increase in ear thickness was measured 24 hr after topical challenge with 0.5% TDI in acetone. Immediately after an intraperitoneal overdose of sodium pentobarbitone the thickness of the TDI-treated ear and the vehicle-treated ear were measured using an

engineers micrometer (Mitutoyo, Japan, No. 293-561) (48). Results are expressed as the difference in ear thickness (Δ ear thickness, mm) between the two ears.

Chemicals

Toluene diisocyanate, olive oil, carbachol were purchased from Sigma Chemical Co. St. Louis, U.S.A. Sodium pentobarbitone was purchased from Sanofi, Maassluis, The Netherlands. Monoclonal antibodies against the different cell subtypes Ly-5-FITC (rIgG2a; CL8990F), Thy1.2-FITC (mIgG2b; CL8600F), L3/T4-FITC (rIgG2b; CL012F), and Ly-2-FITC (rIgG2b; CL169F) and normal rat serum (CL7000) and normal mouse serum (CL8000) were all obtained from CEDARLANE, Ontario, Canada. RPMI 1640 and gentamycin were purchased from Gibco Laboratories, Grand Island NY. Hespan (6% hydroxyethyl starch in 0.9% NaCl, sterile) was purchased from Fresenius AG, Bad Homburg, Germany.

Statistics

All experiments were designed as completely randomized multifactorials with 6-14 mice per group. EC50-and Emax-values for the carbachol-induced tracheal contractions of each experimental animal were calculated separately by nonlinear least-squares regression analysis (simplex minimalization) of the measured contractions vs. carbachol concentration using the sigmoid concentration-response relationship and including a threshold value. The data were analysed by two-way Analysis of Variance followed by a post-hoc comparison between groups. In the figures and tables group means ± sem are given and a difference was considered significant when $P<0.05$. All data manipulation, non-linear fittings, Analyses of Variance and post-hoc comparisons were carried out with a commercially available statistical package (SYSTAT, version 5.03; Wilkinson L. SYSTAT: The system for statistics. Evanston, IL: SYSTAT, Inc., 1990. Statistics).

RESULTS

Pulmonary responses

TDI-sensitization on day 0 and day 1 followed by intranasal challenge on day 8 with 1% TDI caused a significant tracheal hyperreactivity 24 hr after the challenge (*figure 1A*. Emax: nonsensitized 2180 ± 189 mg; TDI-sensitized 2618 ± 51 mg for n=9 mice/group, $P<0.05$). To establish T cell dependency tracheal reactivity was also measured in athymic nude BALB/c mice 24 hr after the challenge. *Figure 1B* clearly demonstrates that there was no difference between the TDI-sensitized and nonsensitized athymic nude mice suggesting an important role for T lymphocytes in the development of TDI-induced tracheal hyperreactivity (Emax: nonsensitized 1421 ± 206 mg; TDI-sensitized 1474 ± 157, for n=4-5 mice/group).

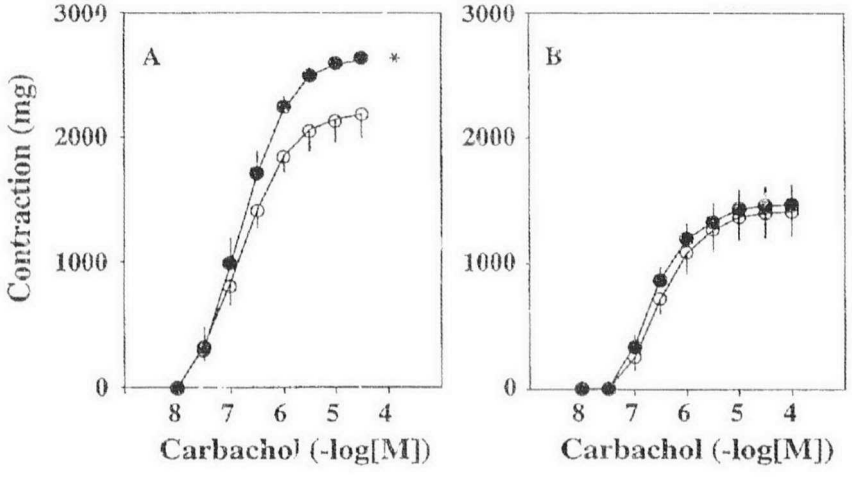

*Figure 1 Tracheal reactivity in (A) BALB/c and (B) BALB/c athymic nude mice. Concentration-response curves to carbachol were measured in the trachea of TDI-sensitized (closed circles) and nonsensitized (open circles) mice 24 hr after the challenge. Results are expressed as mean ± sem for n=4-9 mice/group. Significant differences are denoted by *P<0.05.*

Table 1. Cell percentages in lymphoid tissue and blood in TDI-sensitized and nonsensitized mice after *in vivo* depletion of $CD4^+$ or $CD8^+$ T lymphocytes.

Sensitization	mAb	% $CD4^+$ T cells		% $CD8^+$ T cells	
		blood	lymphoid cells	blood	lymphoid cells
Control	rIgG	45 ± 8	38 ± 5	12 ± 2	16 ± 5
TDI	rIgG	38 ± 8	27 ± 3	8 ± 1	11 ±3
TDI	anti-CD8	45 ± 6	35 ± 5	1 ± 1	3 ± 1
TDI	anti-CD4	6 ± 2	8 ± 3	11 ± 3	19 ± 5
	Depletion	69%	85%	73%	84%

Results are expressed as mean ± sem for n=5-8 mice/group.

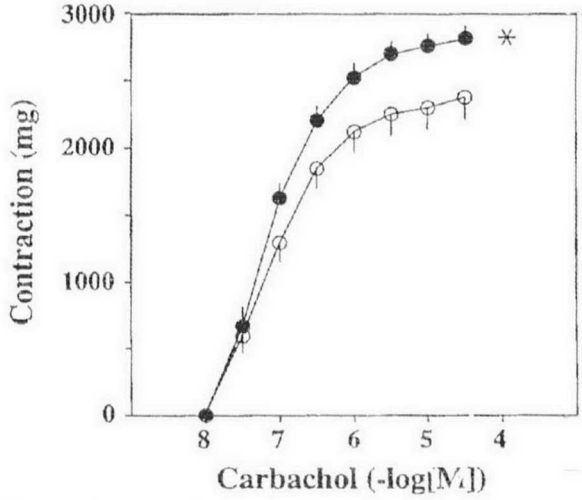

*Figure 2 Adoptive transfer experiments. After adoptive transfer of lymphoid cells from TDI-sensitized and nonsensitized mice, recipient mice were challenged intranasally with 1% TDI. Concentration-response curves to carbachol were measured in the trachea of mice receiving TDI-sensitized lymphoid cells (closed circles) and nonsensitized lymphoid cells (open circles) 24 hr after the challenge. Results are expressed as mean ± sem for n=14 mice/group. Significant differences are denoted by *P<0.05. These results are taken from (274).*

Next, the role of the lymphocytes in TDI-induced tracheal hyperreactivity during the effector phase was investigated. First, adoptive transfer of 5 x 10^6 lymphoid cells from TDI-sensitized donor mice resulted in a significant tracheal hyperreactivity 24 hr after the challenge when compared with transfer of lymphoid cells isolated from nonsensitized mice (*Figure 2*. Emax: Control 2388 ± 167 mg; TDI 2822 ± 91 mg, mean ± sem for n=14 mice/group, P<0.05.). Second, the role of the $CD4^+$ or $CD8^+$ T lymphocytes in TDI-induced tracheal hyperreactivity was further investigated by measuring the effect of *in vivo* depletion of $CD4^+$ and $CD8^+$ T lymphocytes in TDI-sensitized mice. Injection of 100 mg monoclonal antibody on day 3, 4 and 5 in TDI-sensitized mice resulted in a depletion of approximately 70% in lymphoid tissue and 85% in the blood of both $CD4^+$ and $CD8^+$ T lymphocytes (*table 1*). In *figure 3* the tracheal reactivity to carbachol of TDI-sensitized mice *in vivo* depleted of $CD4^+$ or $CD8^+$ T lymphocytes is depicted. Depletion of $CD8^+$ T cells completely inhibited the TDI-induced tracheal hyperreactivity to carbachol whereas the treatment with anti-CD4 antibody did not completely abolish the tracheal

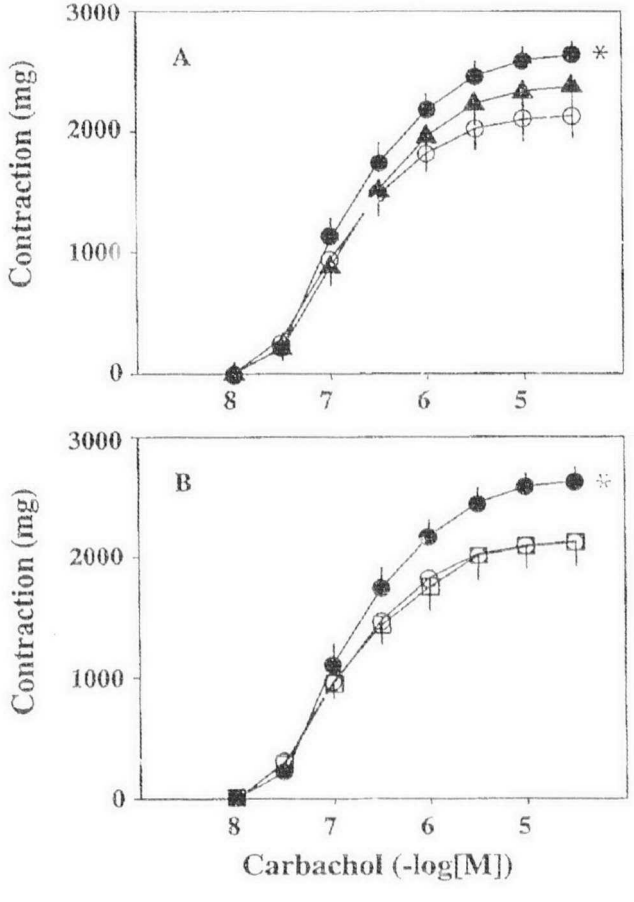

Figure 3 *The effect of in vivo $CD4^+$ T lymphocyte (A) and $CD8^+$ T lymphocyte (B) depletion. TDI-sensitized mice were in vivo depleted of $CD4^+$ or $CD8^+$ T cells by i.p. injection of monoclonal antibodies on day 3, 4, 5 and 6 after sensitization. Concentration-response curves to carbachol were measured in the trachea of nonsensitized (open circles), TDI-sensitized (closed circles), TDI-sensitized depleted of $CD4^+$ T cells (closed triangles) and TDI-sensitized depleted of $CD8^+$ T cells (open squares) mice 24 hr after the challenge. Results are expressed as mean ± sem for n=8-14 mice/group. Significant differences are denoted by $*P<0.05$.*

hyperreactivity in TDI-sensitized mice (*figure 3*). However, a partial reduction was observed after depletion of $CD4^+$ T lymphocytes in TDI-sensitized mice (*figure 3*. Emax: nonsensitized 2167 ± 187 mg; TDI-sensitized 2630 ± 119 mg; TDI-sensitized-$CD8^-$ 2120 ± 209 mg; TDI-sensitized-$CD4^+$ 2365 ± 292 mg, for n=8-14 mice/group).

Table 2. Increase in ear swelling (x 10^{-2} mm) of nonsensitized and TDI-sensitized BALB/c and Athymic nude BALB/c mice 24 hr after the challenge.

Sensitization	BALB/c	Athymic nude BALB/c
Control	0.023 ± 0.005	0.041 ± 0.011
TDI	0.138 ± 0.013*	0.019 ± 0.009

Results are expressed as mean ± sem for n=8-14 mice/group. Significant differences between nonsensitized and TDI-sensitized mice are denoted by *P<0.05.

Cutaneous responses

In addition to pulmonary responses, the cutaneous responses were also determined. Topical challenge with 0.5% TDI caused a significant increase in ear swelling in TDI-sensitized mice when compared with nonsensitized mice (*table 2*). The role of the T lymphocytes in the induction of this DTH-like skin swelling response was ascertained. In agreement with the tracheal responses, the TDI-induced ear swelling 24 hr after the challenge was abolished in athymic nude BALB/c mice (*table 2*). However, in contrast to the pulmonary responses, adoptive transfer of 5 x 10^6 lymphoid cells of TDI-sensitized and nonsensitized donor mice to naive recipients did not lead to an increased ear swelling in the TDI group when compared with the control group (Increased ear swelling: nonsensitized 0.050 ± 0.032 x 10^{-2} mm; TDI-sensitized 0.057 ± 0.027 x 10^{-2} mm, mean ± sem for n=14 mice/group). In addition, the specific contribution of $CD4^+$ and $CD8^+$ T lymphocytes in the TDI-induced ear swelling response during the effector phase was investigated. Interestingly, *in vivo* depletion of $CD4^+$ or $CD8^+$ T lymphocytes by intraperitoneal injection of 100 mg mAb on day 3, 4 and 5, had no effect on the TDI-induced ear swelling 24 hr after the challenge (*table 3*).

DISCUSSION

Previously, we have described a murine model for TDI-induced occupational asthma (274). The characteristics of this model resembled an IgE-independent, delayed-type hypersensitivity (DTH)-like reaction. Skin sensitization followed by intranasal challenge

Table 3. Increase in ear swelling (x 10^{-2} mm) of nonsensitized and TDI-sensitized mice 24 hr after the challenge after *in vivo* depletion of $CD4^+$ or $CD8^+$ T lymphocytes.

Sensitization	mAb	ear swelling (x 10^{-2} mm)
Control	rIgG	0.141 ± 0.022
TDI	rIgG	0.385 ± 0.010*
TDI	anti-CD4	0.341 ± 0.025*
TDI	anti-CD8	0.386 ± 0.022*

Results are expressed as mean ± sem for n=6-11 mice/group. Significant differences between nonsensitized and TDI-sensitized mice are denoted by *, $p<0.05$.

resulted in a nonspecific *in vitro* tracheal hyperreactivity 24 hr after the challenge and no increase in TDI-specific IgE antibodies was found. DTH reactions are T cell-mediated immune responses and in the present study the role of T lymphocytes in the development of the DTH-like TDI-induced tracheal hyperreactivity was investigated. It was established that TDI-induced tracheal hyperreactivity was dependent on T lymphocytes as no difference in tracheal reactivity was observed between TDI-sensitized and nonsensitized athymic nude mice 24 hr after the challenge. Moreover, *in vivo* depletion of $CD4^+$ and $CD8^+$ T lymphocytes revealed that not only $CD4^+$ T cells were involved in TDI-induced tracheal hyperreactivity but that additionally $CD8^+$ T cells have to be considered as DTH effector T cells.

Murine $CD4^+$ T helper cells can be divided into two distinct subsets based on their cytokine secretion profile (210). Th1 cells produce interleukin-2 (IL-2) interferon–γ (IFN–γ) and tumor necrosis factor–β (TNF–β) and are associated with DTH reactions. Th2 cells produce predominantly IL-4, IL-5, IL-6 and IL-10 and are thought to be responsible for several aspects of allergic reactions. Although classically the $CD4^+$ Th1 T lymphocyte is considered to be the DTH effector T cell, studies to define $CD4^+$ or $CD8^+$ T cells as the cellular mediator in DTH reactions have yielded conflicting results. Several studies have indicated that contact hypersensitivity (CHS) responses are mediated by $CD4^+$ T cells (101, 204). In contrast, studies by Gocinski and Tigelaar have demonstrated the ability of both $CD4^+$ and $CD8^+$ T cells to modulate CHS (107). They demonstrated that DNFB-induced ear swelling was enhanced after *in vivo* depletion of $CD4^+$ T cells before sensitization indicating that one $CD4^+$ T cell subset down regulates CHS reactions. In addition, the

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CHAPTER 7

Isolation and biological activity of toluene diisocyanate-specific lymphocyte factors

B 02

Isolation and biological activity of toluene diisocyanate-specific lymphocyte factors

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DNFB-induced ear swelling was reduced but not abolished after *in vivo* depletion of $CD8^+$ T cells, indicating that $CD8^+$ T cells were involved in CHS reactions and that a second $CD4^+$ T cell subset exists with a stimulating function in CHS reactions. Furthermore, Kondo and coworkers used mice lacking the CD4 gene ($CD4^-$) to investigate its role in DNFB-induced ear swelling (146). The CHS response induced by DNFB was reduced but not abrogated in $CD4^-$ mice, indicating that both $CD4^+$ and $CD8^+$ T lymphocytes contributed to DNFB-induced CHS. In contrast with our experiments, both these studies investigated the role of T lymphocyte subsets during the sensitization and challenge phase while in our study T lymphocytes were demonstrated to be involved in the effector phase of the DTH-like TDI-induced tracheal hyperreactivity. Two explanations can be given for the partial inhibition of TDI-induced tracheal hyperreactivity after $CD4^+$ T cell depletion. First, not all $CD4^+$ T cells were depleted and the remaining $CD4^+$ T cell population could have been sufficient to elicit the TDI-induced tracheal response. Second, partial *in vivo* depletion could have resulted in the selective depletion of one of the specific $CD4^+$ T cell subsets with distinct functions during CHS reactions as suggested by Gocinski and Tigelaar (107).

In this present study a prominent role for $CD8^+$ T lymphocytes was demonstrated. Similar to $CD4^+$ Th lymphocytes, $CD8^+$ T lymphocytes can also be divided into two distinct subsets based on their cytokine release profile: Tc1 $CD8^+$ T cells secrete IFN–γ and Tc2 $CD8^+$ T cells secrete IFN–γ, IL-4 and IL-10 (142). Through the release of various cytokines $CD8^+$ T lymphocytes are able to function as T helper cells. Fairchild and coworkers recently described that in DNFB- and oxazolone-induced CHS in mice $CD8^+$ T cells function as effector cells through the production of IFN–γ (320). In comparison with Gocinski and Tigelaar, they found that $CD4^+$ T lymphocytes down regulated the CHS responses by producing IL-4 and IL-10 (320). Moreover, expression of the chemokine interferon–γ inducible protein (IP-10) was abolished after depletion of $CD8^+$ T cells and enhanced after depletion of $CD4^+$ T cells in DNFB-induced CHS reactions (2). These results demonstrated that $CD4^+$ and $CD8^+$ T cells could regulate CHS responses through the release of cytokines.

Generally, however, it is a fair statement that the role of specific cytokines (Th1/Tc1 vs. Th2/Tc2) in the induction of DTH reactions is not well defined. Classically, DTH reactions are thought to be induced by Th1/Tc1 cytokines, such as IFN–γ, and to be down-regulated by Th2/Tc2 cytokines, such as IL-4, IL-5 and IL-10. However, it has been demonstrated that treatment with anti-IL-4 antibodies during the effector phase inhibited trinitrochlorobenzene (TNCB)-induced CHS (268). In addition, preliminary results from

our laboratory using IL-4 knock out mice (supplied by the National Institute of Public Heath and the Environment, Bilthoven, The Netherlands) suggested that IL-4 is also involved in TDI-induced DTH-like reactions. More specifically, 24 hr after the challenge no differences in tracheal reactivity and ear swelling responses were found between TDI-sensitized and nonsensitized IL-4 knock out mice. (Emax: C57/bl 887 ± 141 mg; IL-4 knock out -1 ± 1 mg. D ear swelling C57/bl 0.048 ± 0.015 x 10^{-2} mm; IL-4 knock out 0.012 ± 0.003 x 10^{-2} mm, mean ± sem for n=8-10 mice/group). These results were very exciting but need to be expanded to include IFN–γ and IL-10 knock out mice. These future studies are necessary to define the TDI-induced DTH-like pulmonary response with respect to cytokine profiles.

In addition to the pulmonary response, the role of T lymphocytes was examined in TDI-induced cutaneous responses. In athymic nude BALB/c mice no increase in ear swelling was found after TDI sensitization and challenge. These results demonstrated that T lymphocytes were also required for TDI-induced cutaneous reactions. In contrast, the TDI-induced ear swelling 24 hr after the challenge was not detected after adoptive transfer of 5 x 10^{6} TDI-sensitized lymphoid cells. However, the conditions for these experiments were chosen to induce an increased tracheal reactivity and it could be that different conditions (i.e. amount of transferred cells) would result in an TDI-induced ear swelling. In addition, *in vivo* depletion of $CD4^{+}$ or $CD8^{+}$ T cells failed to exert an effect on TDI-induced ear swelling. These observations could be explained by the fact that a 100% depletion of $CD4^{+}$ or $CD8^{+}$ T cells was never obtained and this could be required for an effect on the TDI-induced cutaneous reaction.

It is interesting to compare the data from our murine study, particularly concerning the role for $CD8^{+}$ T cells, with clinical findings. Our results are consistent with several studies on patients with TDI-induced occupational asthma. For example, the majority of T cell clones, derived from the bronchial mucosa of two patients with TDI-induced asthma were $CD8^{+}$ producing IFN–γ and IL-5 (69). In addition, the late asthmatic reaction in subjects with TDI-induced asthma was associated with an increase in circulating $CD8^{+}$ T cells (83, 163). In conclusion, this present study has demonstrated a role for $CD4^{+}$ and $CD8^{+}$ T lymphocytes in TDI-induced pulmonary responses in the mouse. It is fair to state that T lymphocytes are crucial in the development of these responses but extensive studies are required to ascertain which cytokines are responsible for the changes in airway parameters associated with this model.

B 05

ABSTRACT

Toluene diisocyanate (TDI) is a low molecular weight hapten which is a well known cause of occupational asthma. Recently, we developed a murine model to investigate TDI-induced occupational asthma. Skin sensitization followed by intranasal challenge with 1% TDI resulted in a nonspecific *in vitro* tracheal hyperreactivity 24 hr after the challenge. In addition, the tracheal hyperreactivity could be adoptively transferred with lymphoid cells from TDI-sensitized donor mice (274). This response was IgE-independent and resembled a DTH reaction. In the present study, a TDI-specific lymphocyte factor was isolated from cultured spleen cells of TDI-sensitized mice after 2 days and purified using a TDI-gammaglobulin-sepharose column. Next, the biological activity of this TDI-specific lymphocyte factor was tested in the skin and airways. Sensitization with TDI-factor (60 mg, i.v.) followed by topical application of 0.1% TDI on the ears induced a significant increase in ear swelling 2 hr after the challenge. This response was TDI-specific because topical challenge with 0.5% dinitrofluorobenzene, another low molecular weight hapten, failed to induce an increased ear swelling response in TDI-factor sensitized mice.

With respect to airway responses, mice sensitized with TDI-factor (60 mg, i.v.) exhibited *in vitro* tracheal hyperreactivity to carbachol 2 hr after intranasal challenge with 1% TDI when compared with PBS-sensitized mice. At the same time point (2 hr after the challenge) an increase in mouse mast cell protease-1 (MMCP-1; a specific protease found in mucosal mast cells) was found in the serum and lung tissue of TDI-factor sensitized mice. These results suggested that TDI-specific lymphocyte factors were capable of activating mast cells. Further investigations revealed that the challenge with TDI induced an *in vivo* bronchoconstriction in TDI-factor sensitized mice when compared with PBS-sensitized mice. In conclusion, our results demonstrate that TDI-specific lymphocyte factors are produced after *in vivo* exposure to TDI. These factors are hapten-specific and are biologically active. Moreover, they mimic some of the effects observed after active TDI-sensitization. If these factors are detectable in man, they could be used diagnostically to ascertain whether exposed workers are at risk for developing occupational asthma.

INTRODUCTION

Delayed-type hypersensitivity (DTH) reactions are T cell-mediated immune responses. DTH reactions can be elicited by low molecular weight haptens which are too small to be

antigenic themselves. After the first contact with the hapten, the hapten binds to body proteins thus forming an antigen which can activate two different types of lymphocytes. The first lymphocyte, the DTH-initiating lymphocyte, has been described to have the phenotype Thy-1$^+$, Lyt-1$^+$, CD4$^-$, CD8$^-$, CD3$^-$, IL-2R$^-$, IL-3R$^+$ and B220$^+$ and was therefore suggested to be an antigen specific lymphoid precursor cell that arises before final differentiation to mature T or B lymphocytes (115). The second lymphocyte involved in DTH reactions, the DTH effector T lymphocyte, is Thy-1$^+$, Lyt-1$^+$, CD4$^+$, CD8$^-$, CD3$^+$, IL-2R$^+$, IL-3R$^-$ and B220$^-$ (115). Activation of the DTH-initiating lymphocyte leads to the production of antigen-specific lymphocyte factors which bind to mast cells (150). Upon local second contact with the antigen, the antigen binds to the antigen-specific lymphocyte factors bound to the mast cells, whereby the mast cell is triggered to release its mediators such as serotonin (198). Release of serotonin leads to an increased vascular permeability through activation of 5-HT receptors on the endothelium whereby the extravascular influx of DTH effector T lymphocytes is facilitated. The DTH effector T cells recognize the antigen in the context of MHC class II on antigen presenting cells and are activated to produce cytokines. These cytokines in turn attract leukocytes and mononuclear cells into the tissue which are responsible for the DTH inflammatory response (308).

This cascade of cellular reactions in DTH responses has been examined extensively in the skin using picryl chloride or oxazolone as the sensitizing haptens. In addition, in our laboratories two models were developed to investigate DTH reactions in the lung using picryl chloride and dinitrofluorobenzene (DNFB) as the sensitizing agents. Skin sensitization with DNFB and intranasal challenge with dinitrosulphonic acid caused an increased *in vitro* tracheal reactivity, vascular leakage and accumulation of inflammatory cells in the bronchoalveolar lavage (BAL) fluid (47). In comparison, Garssen and coworkers found an accumulation of mononuclear cells in the lungs of picryl chloride-sensitized mice and airway hyperresponsiveness 48 hr after intranasal challenge with picryl sulphonic acid, a water soluble form of picryl chloride (98). The picryl chloride-induced inflammation of the airways was absent in athymic nude mice, mast cell deficient mice and inhibited by 5-HT receptor antagonists indicating that the responses were T cell and mast cell dependent. (96). The role of the picryl chloride-specific lymphocyte factor was established by inhibition of transferred tracheal hyperresponsiveness after depletion of 14-30 positive cells (monoclonal antibody 14-30 reacts with a common isotype of antigen-specific lymphocyte factors) (95).

More recently, our laboratories have focused on the mechanisms of actions of toluene diisocyanate (TDI), another low molecular weight hapten, which is a well known cause of

occupational asthma. In 80% of the subjects with TDI-induced occupational asthma no increase in TDI-specific IgE antibodies can be found (21, 135, 141). It was therefore suggested that an IgE-independent, DTH-like mechanism could be important for the development of TDI-induced asthma. To investigate this hypothesis we developed a murine model in which skin sensitization on day 0 and day 1 followed by intranasal challenge on day 8 resulted in a nonspecific *in vitro* tracheal hyperreactivity (274). Adoptive transfer of TDI-sensitized lymphoid cells caused a significant tracheal hyperreactivity in the TDI-challenged donor mice when compared with adoptive transfer of nonsensitized lymphoid cells, suggesting a major function of lymphocytes in TDI-induced airway changes (274). Furthermore, in chapter 6 it has been demonstrated by *in vivo* depletion of $CD4^+$ and $CD8^+$ T lymphocytes that both these subsets are involved in the effector phase of TDI-induced DTH-like responses. In the present study, the DTH-like reactions induced by TDI are further investigated by isolating a TDI-specific lymphocyte factor. Moreover, the ability of this factor to mimic some of the effects observed after active sensitization with TDI were examined.

MATERIALS AND METHODS

Animals

Male BALB/c mice (6-8 weeks of age) were supplied either by the Central Animal Laboratory, Utrecht, The Netherlands or by the National Institute of Public Health and the Environment, Bilthoven, The Netherlands. They were housed in groups not exceeding 6 per cage and maintained under standard conditions. All experiments were assessed by the animal ethics committee at Utrecht University and the National Institute of Public Health and the Environment.

Sensitization procedure

Mice were sensitized twice daily on day 0 and day 1 with 1% TDI dissolved in acetone:olive oil (4:1) which was applied epicutaneously to the shaved abdomen and thorax (100 µl) and four paws (100 µl) under light anaesthesia (sodium pentobarbitone; 50 µl; 30 mg/kg i.p.).

Isolation and purification of toluene diisocyanate-specific lymphocyte factors

On day 5 TDI-sensitized mice were sacrificed by cervical dislocation and the spleens were isolated under sterile conditions. Cell suspensions were made by gently dispersing the cells from the spleen through a filter using the back of a syringe. After washing the spleen cells 3 times, cells were cultured for 2 days at a density of 10×10^6 cell/ml in RPMI 1640 supplemented with 10 mM 2-mercaptoethanol, 100 IU/ml penicillin and 50 mg/ml streptomycin, at 37°C, 5% CO_2 in a humidified atmosphere. Hereafter the culture

supernatant was collected and a TDI-specific lymphocyte factor was isolated by hapten-affinity chromatography using a TDI-gammaglobulin-sepharose column. After washing the column with phosphate buffered saline (PBS, pH=7.2), the TDI-specific lymphocyte factor was eluted with 5 M guanidine. The eluate was dialysed extensively against PBS at 4°C.

SDS-polyacrylamide gelelectrophoresis of TDI-lymphocyte factor
Proteins of the TDI-factor were taken up in reducing Laemmli sample buffer which contained 100 mM dithiotreitol. Samples were boiled for 3 minutes and subsequently the proteins were fractionated on a 12.5% polyacrylamide gel. Following gel electrophoresis, proteins were visualized by silver staining (Biorad, The Netherlands).

Sensitization with the isolated TDI-specific lymphocyte factor
Mice were injected i.v. in the retroorbital plexus with 100 ml TDI-factor (6 mg/ml) or PBS under light anaesthesia (sodium pentobarbitone; 50 µl; 30 mg/kg i.p.). In additional experiments the same doses of TDI-factor or PBS were injected under light ether anaesthesia and the thickness of both ears was measured simultaneously.

Challenge procedures
Thirty minutes after injection of the TDI-factor or PBS mice were challenged intranasally with 1% TDI dissolved in ethyl acetate:olive oil (1:4). Twenty µl of the TDI solution was applied intranasally under light anaesthesia (sodium pentobarbitone; 50 µl; 30 mg/kg i.p.). Furthermore, mice were also challenged on the ears; TDI (20 µl; 0.5%; dissolved in acetone) or vehicle control (20 µl) were applied topically to both sides of the ears in experiments using sodium pentobarbitone anaesthesia. In additional experiments, light ether anaesthesia was used and both ears were challenged with 40 ml 0.1% TDI dissolved in acetone:olive oil (4:1). To test the hapten-specificity of the TDI-specific lymphocyte factor TDI-factor and PBS-treated mice were also topically challenged on the ears with 40 ml 0.5% DNFB dissolved in acetone.

Measurement of cutaneous reactions
An increase in ear thickness was measured 2 hr after topical challenge with 0.5% or 0.1% TDI. The thickness of the TDI-treated ears were measured using an engineers micrometer (Mitutoyo, Japan, No. 293-561) under light sodium pentobarbitone (50 µl; 30 mg/kg i.p.) and ether anaesthesia. Results are expressed as the difference in ear thickness (Δ ear thickness, mm) between the left and right ear when sodium pentobarbitone was used or as the difference between the ears before challenge and after the challenge when light ether anaesthesia was used (48).

Isometric measurement of tracheal reactivity
Two hr after intranasal challenge with 1% TDI tracheal reactivity was measured using the method of Garssen et al. (97). Mice were killed by an overdose of sodium pentobarbitone (0.3 ml; 60 mg/kg i.p.). The trachea, which was resected *in toto*, was carefully cleaned of connective tissue using a binocular microscope. A 9 ring piece of the trachea (taken from just below the larynx) was then transferred to a 10 ml organ bath containing a modified oxygenated Krebs solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCL_2 6H_2O$, 0.5 mM

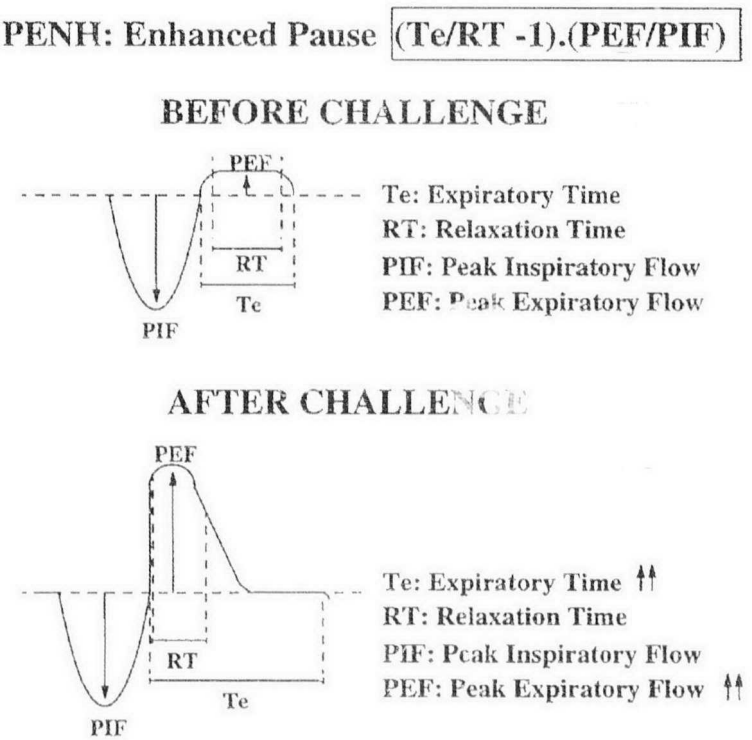

Figure 1 Explanation of the Enhanced Pause (FENH). PENH is the lung function parameter developed by BUXCO Electronics, which is a indication for bronchoconstriction.

4°C, the plates were washed again 5 times with PBS/Tween 20 and incubated for 30 minutes at room temperature with 50 µl o-phenylenediamine dihydrochloride (2.2 mM in 0.1 M citric/phosphate buffer, pH 5.0). The reaction was stopped by adding 25 µl 2.5 M H_2SO_4 and the absorption was measured at 492 nm using a microplate reader.

Chemicals

Toluene diisocyanate, olive oil, carbachol, penicillin, streptomycin and o-phenylenediamine dihydrochloride were purchased from Sigma Chemical Co. St. Louis, U.S.A. Sodium pentobarbitone was purchased from Sanofi, Maassluis, The Netherlands. RPMI 1640 and 2-mercaptoethanol were purchased from Gibco Laboratories, Grand Island, NY. Hespan (6% hydroxyethyl starch in 0.9% NaCl, sterile) from Fresenius AG, Bad Homburg, Germany. Guanidine was purchased from Boehringer Mannheim, Almere, The Netherlands.

B 10

Statistics

All experiments were designed as completely randomized multifactorials with 3-9 mice per group. EC50-and Emax-values for the carbachol-induced tracheal contractions of each experimental animal were calculated separately by nonlinear least-squares regression analysis (simplex minimalization) of the measured contractions vs. carbachol concentration using the sigmoid concentration-response relationship and including a threshold value. The data were analysed by two-way Analysis of Variance followed by a post-hoc comparison between groups. In the figures and tables group means ± sem are given and a difference was considered significant when $P<0.05$. All data manipulation, non-linear fittings, Analyses of Variance and post-hoc comparisons were carried out with a commercially available statistical package (SYSTAT, version 5.03; Wilkinson L. SYSTAT: The system for statistics. Evanston, IL: SYSTAT, Inc., 1990. Statistics).

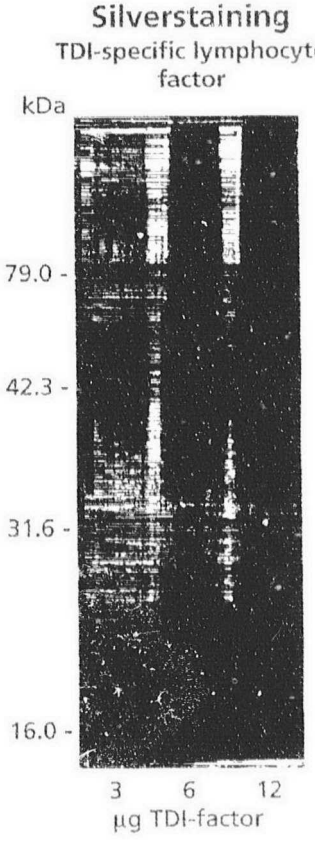

Figure 2 Protein pattern of TDI-specific lymphocyte factor. TDI-specific lymphocyte factor (3, 6 and 12 mg) were fractionated on a 12.5% polyacrylamide gel. Proteins were visualized by silver staining (see materials and methods).

RESULTS

Isolation and purification of a TDI-specific lymphocyte factor

A TDI-specific lymphocyte factor was isolated from the culture supernatant of TDI-sensitized spleen cells. The TDI-specific lymphocyte factor was eluted from a TDI-gammaglobulin-sepharose column with 5 M guanidine and the protein content was determined by a spectrophotometer. This procedure was used to ascertain that no intact immunoglobulin was present. Hapten-specific lymphocyte factors can resist the denaturating isolation conditions, whereas immunoglobulins can not (personal communication Dr. F. Redegeld). The TDI-specific lymphocyte factor was fractionated on a polyacrylamide gel to determine the protein contents. In *figure 2* the silverstained polyacrylamide gel of the TDI-factor (3, 6 and 12 mg TDI-factor) is depicted. It is clear that the TDI-specific lymphocyte factor exists as a complex of different proteins.

Biological activity of the TDI-specific lymphocyte factor

Measurement of cutaneous reaction

The increase in ear thickness was first measured 2 hr after topical challenge with 0.5% TDI in acetone using light sodium pentobarbitone anaesthesia. However, this procedure did not result in a difference between TDI-factor sensitized and PBS-treated mice (TDI-factor $0.011 \pm 0.006 \times 10^{-2}$ mm; PBS-treated $0.011 \pm 0.006 \times 10^{-2}$ mm, mean ± sem for n=3 mice/group). Hereafter, the increase in ear thickness was measured 2 hr after topical challenge with 0.1% TDI in acetone:olive oil (4:1) using light ether anaesthesia. Mice injected with the TDI-factor exhibited significantly more increase in ear swelling when compared with mice injected with PBS (*figure 3*). Furthermore, topical challenge with 0.5% DNFB of TDI-factor and PBS injected mice did not cause an increased ear swelling in the TDI-factor treated mice (increased ear thickness: PBS $0.170 \pm 0.018 \times 10^{-2}$ mm; TDI-factor $0.113 \pm 0.019 \times 10^{-2}$ mm, mean ± sem for n=5 mice/group, $P>0.05$). This indicated that the TDI-factor-induced increased ear swelling was specific for TDI.

Measurement of tracheal reactivity

The biological activity of the TDI-factor was also assessed by measuring the *in vitro* tracheal reactivity to carbachol. In *figure 4* it is clearly demonstrated that the TDI-factor was capable of inducing tracheal hyperreactivity 2 hr after the challenge. The Emax (maximal contractile response) induced by carbachol of trachea taken from TDI-factor sensitized mice was significantly higher when compared with trachea taken from PBS-

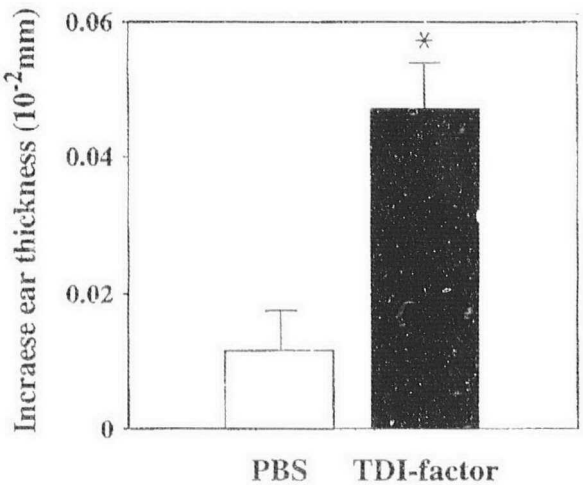

*Figure 3 Ear swelling response 2 hr after the challenge. PBS (open bar) and TDI-factor (closed bar) treated mice were topically challenged with 0.1% TDI. The increase in ear swelling was measured 2 hr after the challenge using a micrometer. The difference (x 10^{-2} mm) between the ears before and after the challenge is expressed as mean ± sem for n=7 mice/group. Significant differences are indicated by *P<0.05.*

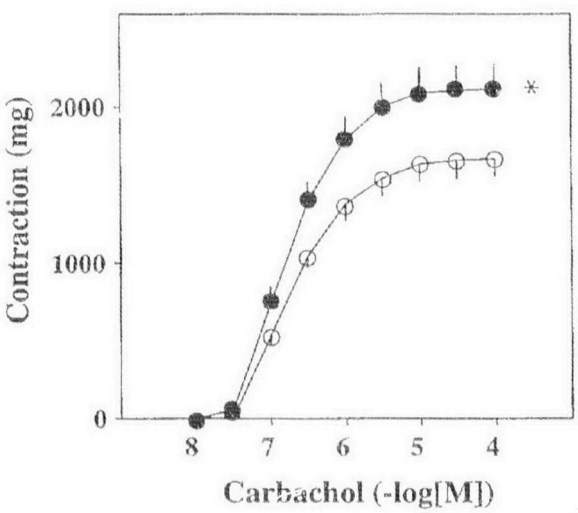

*Figure 4 Tracheal reactivity 2 hr after the challenge with 1% TDI. Concentration-response curves to carbachol (10^{-8}-10^{-4} M) were measured in the trachea of PBS-treated (open circles) and TDI-factor sensitized (closed circles) mice 2 hr after the challenge. Results are expressed as mean ± sem for n=8-9 mice/group. Significant differences are indicated by *P<0.05.*

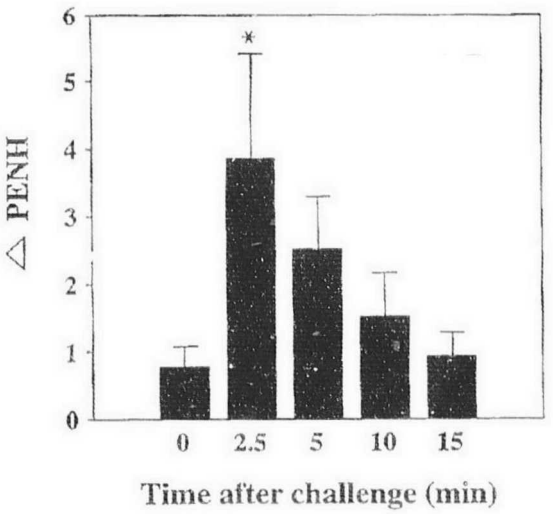

Figure 5 Direct in vivo bronchoconstriction in TDI-factor sensitized mice. PENH was recorded in PBS-treated and TDI-factor sensitized mice for 15 minutes after the challenge. Results are expressed as the difference in PENH between PBS-treated and TDI-factor sensitized mice, for n=3 mice/group.

treated mice (Emax: PBS 1670 ± 114 mg; TDI-factor 2115 ± 164 mg, mean ± sem for n=8-9 mice/group, $P<0.05$). In contrast, no difference in sensitivity (EC50) to carbachol was detected between the PBS and TDI-factor sensitized mice (EC50: PBS 0.179 ± 0.022 x 10^{-6} M; TDI-factor 0.153 ± 0.016 x 10^{-6} M, mean ± sem for n=8-9 mice/group, $P>0.05$).

Measurement of in vivo bronchoconstriction

Immediately after the challenge TDI-factor sensitized and PBS-treated mice were placed in a body plethysmograph to measure the PENH which is an arbitrary unit for bronchoconstriction (*figure 1*). The mice were conscious and the PENH was measured during 15 minutes after the challenge. Immediately after the challenge (2.5 minutes) a significant difference between TDI-factor sensitized and PBS-treated mice was observed (PENH: PBS 3.15 ± 0.73; TDI-factor 7.01 ± 1.18, mean ± sem for n=3 mice/group, $P<0.05$). In *figure 5* the difference in PENH between the PBS-treated and TDI factor sensitized mice for 15 minutes is depicted. This direct bronchoconstriction response was resolved 15 minutes after the challenge (*figure 5*).

B 14

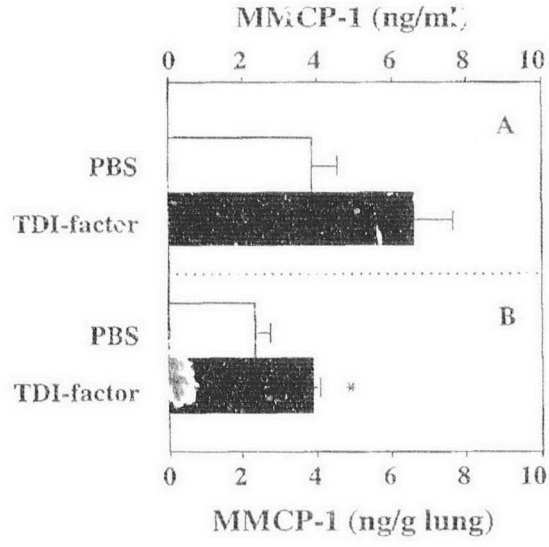

*Figure 6 Mouse mast cell protease-1 (MMCP-1) levels in the serum and lung tissue. Levels of MMCP-1 were measured in the serum and lung tissue of PBS-treated (open bars) and TDI-factor sensitized (closed bars) mice 2 hr after the challenge using a standard ELISA kit. Results are expressed as mean ± sem for n=4-5 mice/group. Significant differences are indicated by *P<0.05.*

Measurement of mouse mast cell protease-1 (MMCP-1)

MMCP-1 was measured in the lung tissue and serum 30 minutes and 2 hr after intranasal challenge with TDI in PBS and TDI-factor sensitized mice. No differences were measured between the PBS and TDI-factor sensitized mice in the serum and lung tissue 30 minutes after the challenge (MMCP-1, serum: PBS 8.43 ± 2.38 ng/ml; TDI-factor 6.69 ± 2.25 ng/ml. MMCP-1, lung: PBS 3.12 ± 0.54 ng/g lung; TDI-factor 3.22 ± 0.23 ng/g lung, mean ± sem for n=4-5 mice/group, P>0.05). In *figure 6* the results from the MMCP-1 levels 2 hr after the challenge in the serum and lung tissue are presented. Two hr after the challenge marked differences were found in MMCP-1 levels between the lung tissue and serum of TDI-factor sensitized mice when compared with PBS-treated mice (*figure 6*).

DISCUSSION

In the present study we isolated and purified a protein complex produced by spleen cells of TDI-sensitized mice. This protein complex proved capable of inducing TDI-specific ear swelling, *in vitro* tracheal hyperreactivity and mast cell activation 2 hr after TDI challenge and a direct bronchoconstriction response 2.5 minutes after TDI challenge. From literature it is known that antigen-specific lymphocyte factors are produced within 1 to 2 days after sensitization and are responsible for the early phase associated with DTH reactions (115). In our active sensitization model for TDI-induced occupational asthma no early responses were detected, as measured by *in vitro* tracheal hyperreactivity to carbachol and the cutaneous ear swelling response (272). However, in the present study it appeared that light sodium pentobarbitone anaesthesia inhibited the TDI-induced ear swelling in TDI-factor sensitized mice whereas with light ether anaesthesia a significant ear swelling could be measured. In agreement with our results, Veronesi and coworkers recently described that the DNFB-induced ear swelling in sodium pentobarbitone anaesthetized mice was suppressed 62% and 76% at 4 and 24 hr postexposure when compared with DNFB-treated unanesthetized mice (310). It may therefore be possible that in our active sensitization model we were unable to ascertain an early response using sodium pentobarbitone anaesthesia.

In the IgE-mediated long exposure model for TDI-induced occupational asthma an early response, both ear swelling and *in vitro* and *in vivo* airway hyperresponsiveness, was detected (chapter 3). In these experiments sodium pentobarbitone anaesthesia was used and seemed to have no inhibitory effect on the responses. These results imply that there are differences between IgE-mediated and T cell-dependent early responses. Van Loveren and coworkers have investigated the different mechanisms of IgE-dependent and T cell-dependent early responses (308). They found a difference between mast cell mediator release. More specifically, the T cell-dependent reactions induced release of serotonin from non-granular sites in the cytoplasm, whereas IgE-dependent reactions induced mediator release from the granules of mast cells resulting in not only serotonin but also histamine release. These observations were further confirmed by morphological studies showing massive mast cell degranulation in IgE-dependent reactions but more subtle changes in the ultrastructure of mast cells in T cell-dependent reactions (11). The conclusion from these studies is that the IgE-mediated TDI-induced early responses are mechanistically different from the TDI-specific lymphocyte factor-induced early changes.

C 02

CHAPTER 8

Summary and conclusions

C 03

Summary and conclusions

The experiments presented in this study shed more light on the effects of exposure to TDI. Although they just start to reveal the cascade of immunological reactions, the results are interesting and deserve further investigation. Of particular interest was the bronchoconstriction response which was observed in TDI-factor sensitized mice immediately after the challenge. In subjects with TDI-induced occupational asthma immediate, late and dual asthmatic responses have been recorded, although isolated immediate asthmatic reactions are scarce (141, 208, 303, 306). It is a plausible hypothesis that the immediate asthmatic reaction in subjects with IgE-independent TDI-induced asthma is evoked by TDI-specific lymphocyte factors. The ability of TDI-specific lymphocyte factor to arm and facilitate the activation of mast cells was also an important finding and this interaction will be further established using mast cell deficient mice.

In conclusion, the data presented in this study, demonstrates the presence and biological activity of a TDI-specific lymphocyte factor. This result certainly is in agreement with the hypothesis that a DTH-like reaction is involved in occupational asthma. It is a fact that in only 20% of the subjects with TDI-induced occupational asthma an increase in TDI-specific IgE antibodies can be found. Therefore, routine serologic screening of workers for TDI-specific IgE antibodies is irrelevant in providing an early warning of developing TDI hypersensitivity. For other types of isocyanates (HDI and MDI) serum levels of specific IgG antibodies were more satisfactory in their association with occupational asthma (52). It is our proposal that antigen-specific lymphocyte factors could be used to screen exposed workers to ascertain whether they are at risk for developing occupational asthma. Moreover, early removal of the subjects positive for antigen-specific lymphocyte factor from their workplace may lessen the severity or prevent the further development of occupational asthma.

C 05

Occupational asthma has become an increasing problem in the developed countries in the last decade. Already 200 known causes of occupational asthma have been identified and it is generally thought that more causes will be recognized in the future. Agents causing occupational asthma can be divided into two groups: the high molecular weight allergens and the low molecular weight haptens, which are too small to be antigenic themselves and will bind to body proteins. The high molecular weight allergens, mostly proteins derived from flour, animal-derived antigens and enzymes, cause occupational asthma via an allergic IgE-dependent mechanism. The second and largest group of agents causing occupational asthma, the low molecular weight haptens, such as isocyanates, wood dusts and amines, cause occupational asthma through a largely unknown mechanism. The first striking dissimilarity between allergic IgE-dependent asthma and low molecular weight compound-induced asthma is that only a small portion of subjects have an increase in hapten-specific IgE antibodies. The precise percentage of subjects with increased IgE antibodies depends on the occupational agent. For example, in isocyanate-induced asthma approximately 20% of the subjects have an increase in serum IgE antibodies, whereas for amine-induced asthma this population is considerably higher at almost 50%. This division of subjects with low molecular weight compound-induced asthma into two distinct groups suggests that these agents are capable of inducing occupational asthma through more than one immunological mechanism.

Isocyanates are the most prevalent group of low molecular weight haptens causing occupational asthma. Toluene diisocyanate (TDI) is extensively used in industries, mainly in the production of paints and polyurethane foams. Approximately 5-10% of the exposed workers develop occupational asthma. These subjects exhibit a nonspecific bronchial hyperresponsiveness and an inflammation of the airways, characterized by T lymphocytes (both $CD4^+$ and $CD8^+$), neutrophils, eosinophils and mast cells. Screening of TDI-sensitized subjects is primarily performed using serologic tests for TDI-specific IgE antibodies. However, this will identify only 20% of the subjects with TDI-induced asthma. The majority of the TDI-sensitized subjects missed by this test, will continue working and continue being exposed to TDI which will eventually lead to permanent, persistent asthma. Therefore it is extremely important to know and understand more about the mechanism of action of TDI. The initial aim of this thesis was to develop two models in the mouse to investigate the various aspects of TDI-induced occupational asthma (i.e. IgE-independent and IgE-mediated). In literature several models for TDI-induced asthma are described and the majority of these models are in guinea pigs. These models have focused primarily on the IgE-dependent mechanism of TDI-induced asthma. In this thesis, a different approach

was taken to investigate TDI-induced occupational asthma. The research presented in this thesis focused on the participation of IgE-independent mechanisms in the development of TDI-induced occupational asthma.

Pulmonary responses

First in chapter 2, an IgE-independent model for TDI-induced asthma is developed. In our laboratories two models for IgE-independent asthma have already been developed using the low molecular weight compounds picryl chloride and dinitrofluorobenzene (DNFB) as sensitizing haptens. Picryl chloride and DNFB-induced pulmonary changes in the mouse were demonstrated to be cell-mediated immune reactions. Delayed-type hypersensitivity (DTH) reactions are cell-mediated IgE-independent immune reactions, classically investigated in the skin and easily elicited by low molecular weight compounds. Upon first contact (sensitization phase) the hapten binds to body proteins and activates DTH-initiating lymphocytes which start to produce hapten-specific lymphocyte factors. These lymphocyte factors bind to mast cells, macrophages and possibly other cells. Local second contact with the antigen (challenge phase) leads to binding of the antigen to the hapten-specific lymphocyte factors bound to mast cells. Subsequently, the mast cell is triggered to release vasoactive mediators, such as serotonin. Serotonin causes an increased vascular permeability leading to the infiltration of circulating antigen-specific DTH effector T cells. These DTH effector T cells can recognize antigen extravascularly in the context of major histocompatibility (MHC) class II on antigen presenting cells (APC) after a second contact with the antigen. The DTH effector T cells are hereby triggered to produce cytokines leading to a DTH response.

Mice were skin sensitized twice daily on day 0 and day 1 with 1% TDI on their shaved abdomen and four paws. Next, on day 8 mice were intranasally challenged with 1% TDI. This exposure regime to TDI did not result in a detectable level of TDI-specific IgE antibodies, indicating that it is highly unlikely that IgE antibodies play a role in this reaction. This result was not so surprising considering the production kinetics for IgE antibodies; it is generally accepted that 10-12 days are needed to generate IgE antibodies. In this model, *in vitro* tracheal reactivity was measured in TDI-sensitized and nonsensitized (vehicle treated) mice. Twenty four hr after the challenge TDI-sensitized mice exhibited *in vitro* tracheal hyperreactivity to carbachol, a muscarinic receptor agonist, and to serotonin when compared with nonsensitized mice. Moreover, 24 hr after the challenge this tracheal hyperreactivity was associated with an increase in myeloperoxidase (MPO, present in neutrophil granules) activity, suggesting an effect of TDI on neutrophil activity. However,

using light microscopy no influx of leukocytes could be detected histologically in the trachea, lung tissue and airway lumen of TDI-sensitized mice. Therefore it was suggested that the increase in MPO activity 24 hr after the challenge should be interpreted as an increase in MPO content per neutrophil, representing a different activation state in TDI-sensitized and nonsensitized mice.

This mouse model for TDI-induced occupational asthma exhibited most of the characteristics of TDI-induced asthma: no increase in TDI-specific IgE antibodies, nonspecific tracheal hyperreactivity and an increase in MPO levels suggesting the involvement of neutrophils. It was postulated that this murine model for TDI-induced asthma could be used to investigate the IgE-independent DTH-like immunological mechanisms of TDI. Next, preliminary experiments were performed to establish a role for lymphocytes in the induction of DTH-like TDI-induced tracheal hyperreactivity. Lymphocytes isolated from TDI-sensitized mice were able to adoptively transfer tracheal hyperreactivity to naive recipients, suggesting that a cell-mediated DTH-like mechanism could be involved in TDI-induced tracheal hyperreactivity in the mouse. Of course, adoptive transfer of a mixed lymphocyte population (containing $CD4^+$ T cells, $CD8^+$ T cells, B cells, mast cells, basophils, macrophages) did not establish an exclusive role for T lymphocytes in the induction of tracheal hyperreactivity, but the DTH-like reaction induced by TDI was further validated in chapters 5, 6 and 7 of this thesis.

In the following chapter (chapter 3) it was demonstrated that TDI was also able to induce another type of immunological reaction; an IgE-mediated model for TDI-induced asthma was developed. Repeated topical application of TDI, once weekly for 6 consecutive weeks (long exposure protocol), resulted in the production of TDI-specific IgE and IgG antibodies. The induction of IgE antibodies was associated with a different pattern of pulmonary changes when compared with the IgE-independent model (short exposure protocol, chapter 2). TDI-sensitization according to the long exposure protocol resulted in *in vitro* tracheal hyperreactivity 3 hr after the challenge (1% TDI intranasally, week 7). Moreover, 3 hr after the challenge *in vivo* airway hyperresponsiveness to serotonin was found in TDI-sensitized mice. Therefore in this model, not only was the presence of TDI-specific IgE antibodies established but this feature was associated with much earlier changes (3 hr after the challenge) in airway function when compared with the IgE-independent short exposure model. The production of TDI-specific IgE antibodies is an important link to allergic asthma in man, however, the strict IgE-dependency of the airway hyperresponsiveness observed in the long exposure model will have to be determined in future studies. Regardless of this reservation, it is a safe assumption that the mechanisms

responsible for the development of TDI-induced pulmonary changes were different in the short and long exposure models.

IgE-mediated responses and IgE-independent DTH reactions are regulated by different T lymphocyte subsets. $CD4^+$ T helper cells have been divided into two distinct subsets based on their cytokine secretion profile. Th1 lymphocytes produce interleukin-2 (IL-2), interferon–γ (IFN–γ) and tumor necrosis factor–β (TNF–β) and are associated with DTH reactions. Th2 lymphocytes produce IL-4, IL-5, IL-6 and IL-10 and are involved in allergic IgE-dependent mechanisms. In general, several factors that push a T cell response towards a predominantly Th1 or Th2 phenotype have been proposed, including the properties of antigens, dose of antigen, site of exposure and ongoing immune response in the host (261). For example, it has been demonstrated in mice that infection with a low number of parasites (*Leishmania major*) induced cell-mediated immunity whereas high dose infection resulted in a parasite-specific antibody, Th2-like response (200). *In vitro* studies using synthetic peptides also indicated the importance of antigen dose for the development of Th1 or Th2 responses, however contradictory data are published (64, 120). Furthermore, it has been demonstrated that the type of APC regulates the balance between Th1 and Th2 (79, 89). Interestingly, it was shown that repeated topical application of trinitrochlorobenzene (TNCB, a contact sensitizing agent) to the ears of mice induced immediate-type (Th2) ear swelling, in contrast to more delayed-type (Th1) ear swelling after single application (143). Unfortunately, studies among subjects with TDI-induced occupational asthma failed to find a similar association between the length of exposure and the type of response elicited by TDI. This negative finding is probably not so surprising after considering the heterogeneity of subjects with TDI-induced asthma. It has already been suggested that genetic factors play a role in the development of TDI-induced asthma. Furthermore, age, sex, length of employment, smoking habits, stress, history of atopy, exposure time, concentration of TDI exposed to and type of exposure are all likely to influence the development and type of TDI-induced asthma. In conclusion, in this chapter it is clearly demonstrated that short and long term topical exposure to TDI resulted in IgE-independent and IgE-mediated responses indicating that TDI is capable of inducing different types of immunological reactions. Such differential actions of TDI could explain the diversity of symptoms found in subjects with TDI-induced occupational asthma.

In literature several models for TDI-induced occupational asthma have been described, but the majority of these models focus on IgE-dependent reactions. The two models described in chapter 2 (IgE-independent) and chapter 3 (IgE-mediated) can both be used to clarify the mechanism of action of TDI. In this thesis a choice was taken and further

investigations focused on the IgE-independent DTH-like short exposure model. This cause of action was chosen because the cell-mediated immune response elicited by TDI is highly overlooked and the majority of subjects with TDI-asthma did not exhibit TDI-specific IgE antibodies.

In DTH reactions mast cells and T lymphocytes play an important role. Recent studies have clearly demonstrated that these two cell types can be regulated by sensory neuropeptides. Sensory neuropeptides are stored in the sensory nerves which innervate the airways and are in close contact with cells of the immune system. Interestingly, it was demonstrated *in vitro* that TDI could directly activate capsaicin-sensitive sensory nerves in rat isolated urinary bladder and guinea pig bronchi (179, 182). Furthermore, it has been demonstrated that sensory neuropeptides, especially the tachykinins substance P (SP), neurokinin A (NKA) and NKB, play a role in the development of pulmonary DTH reactions induced by the low molecular weight hapten DNFB (47). In chapter 4 the effect of TDI on the sensory nerves in the mouse airways was investigated. First, it was demonstrated *in vitro* that TDI was capable of relaxing tracheal smooth muscle. This TDI-induced relaxation was dose-dependently inhibited by preincubation with the NK_1 tachykinin receptor antagonist, RP 67580. It was concluded that TDI facilitated the release of tachykinins from sensory nerves, which activated the NK_1 receptor resulting in a relaxation of the isolated mouse trachea. Subsequently, the involvement of sensory neuropeptides in the development of TDI-induced tracheal hyperreactivity after the short exposure protocol was investigated. Both capsaicin pretreatment (which leads to the depletion of sensory neuropeptides) and treatment with the NK_1 receptor antagonist, RP 67580, resulted in the abolition of TDI-induced tracheal hyperreactivity 24 hr after the challenge. Interestingly, the NK_1 receptor antagonist only inhibited the TDI-induced hyperreactivity when administered before the challenge; administration during the sensitization phase had no effect. These results clearly indicated that sensory neuropeptides are essential for the development of TDI-induced tracheal hyperreactivity during the effector phase.

The next question was how are the sensory neuropeptides regulating the tracheal hyperreactivity during the effector phase? A direct action of the tachykinins on the NK_1 receptor in the mouse airway smooth muscle is not a likely answer to this question, because in the mouse tachykinins relaxed the airways and this response would not lead to an increased tracheal reactivity. Therefore an interaction of neuropeptides with cells of the immune system, such as mast cells, T lymphocytes, macrophages or a combination of these cell populations was postulated. It has been demonstrated that tachykinins can

concentration-dependently enhance the proliferation of mitogen-stimulated T lymphocytes (56, 234, 287). In addition, SP has been reported to activate macrophages (145), induce the production of cytokines (162) and facilitate the migration of macrophages, neutrophils and T lymphocytes (187, 207). In chapter 2 it was proposed that lymphocytes and neutrophils are involved in TDI-induced tracheal hyperreactivity and it is an interesting hypothesis that this could be due to activation by tachykinins released during the effector phase. Moreover, it has been demonstrated that SP activates mast cells of the respiratory system (114) resulting in the release of bioactive mediators. It is therefore possible that intranasal challenge with TDI resulted in the release of tachykinins which subsequently activated mast cells.

Indeed in the next chapter (chapter 5) the role of the mast cell in the TDI-induced airway responses was examined. It has already been established that in subjects with TDI-induced asthma mast cells are activated in the bronchial epithelium (262, 288). In the present study, mast cell activation was detected by measuring increased levels of mouse mast cell protease-1 (MMCP-1), which is exclusively present in granules of mucosal mast cells. MMCP-1 levels were increased in the serum and lung tissue of TDI-sensitized mice at all measured time points after and even before the challenge. The conclusion from these results is that TDI-sensitization alone is capable of inducing mast cell activation in the mouse airways. The next important question was whether this mast cell activation was related to the observed tracheal hyperreactivity. Until now, no studies have been performed in subjects with TDI-induced asthma or in animal models for TDI-induced occupational asthma to correlate mast cell activation with airway hyperresponsiveness. In this thesis, the role of TDI-induced mast cell activation in the development of tracheal hyperreactivity was investigated. Mast cell deficient (W/W^v) mice and their normal control littermates were sensitized and challenged according to the short exposure protocol. Interestingly, 24 hr after the challenge there was a significant difference in tracheal reactivity to carbachol between the TDI-sensitized and nonsensitized W/W^v mice as well as in their normal control littermates. The obvious conclusion from these results is that mast cells are not involved in TDI-induced tracheal hyperreactivity. However, in literature some uncertainty exist concerning the extent of mast cell deficiency in W/W^v mice. Indeed, in mast cell deficient mice a small number of mast cells are still present (9) and it has been postulated that as little as 1% of the total mast cell population is sufficient for initiation of DTH reactions (248). In addition, it has been demonstrated that repeated application of TNCB to the skin of W/W^v mice induced the presence of mast cells (143). Whether this was *de novo* production or migration of mast cells was not discussed in this article.

A role for mast cell mediators in TDI-induced responses was further investigated in chapter 5. The contribution of serotonin and histamine, two mast cell mediators, during the effector phase was investigated by treatment with ketanserin, a serotonin $(5\text{-HT})_2$ receptor antagonist, and cimetidine, a histamine H_2 receptor antagonist. Interestingly, no differences in tracheal reactivity were observed between ketanserin (16 mg/kg, i.p.)-treated TDI-sensitized mice and ketanserin-treated nonsensitized mice 24 h after the challenge, suggesting that serotonin plays an important role in TDI-induced tracheal hyperreactivity. These results are in agreement with other findings observed in pulmonary DTH reactions. Garssen and coworkers demonstrated that the picryl chloride-induced accumulation of mononuclear cells in the mouse airways was serotonin dependent (96). In addition, Askenase and coworkers performed extensive research on the role of serotonin in cutaneous DTH responses. Cutaneous DTH responses were demonstrated to be dependent on the early release of serotonin from mast cells, which facilitated the influx of DTH effector T cells. Interestingly, the role of serotonin in DTH responses became more evident when mast cell deficient mice were used. In W/W^v mice the cutaneous DTH response was still detectable but could be inhibited by 5-HT receptor antagonists (102). Furthermore, it was demonstrated that depletion of platelets abolished the DTH reaction in W/W^v mice (102). It was hypothesized that in W/W^v mice serotonin is released from platelets and that this action is a compensatory mechanism for the lack of mast cells which normally release large amount of serotonin.

In addition, administration of cimetidine (16 mg/kg, i.p.) around the time of challenge abolished the TDI-induced tracheal hyperreactivity, suggesting that histamine also plays a role in the effector phase of TDI-induced tracheal hyperreactivity through activation of the H_2 receptor. The exact source of histamine in TDI-induced pulmonary changes is unclear in light of the experiments performed in chapter 6. Histamine is an important mediator contained in mast cells, however, it is also present in basophils and neurons. In addition to the source, the location of the H_2 receptor involved in this response is also uncertain. H_2 receptors are located on many cells, including T cells, mast cells and their presence is even suggested on eosinophils (30, 249, 312). The H_2 receptors on these immune cells are able to modulate the release of bioactive mediators. Until now, no studies have been published investigating the role of histamine and H_2 receptors in the effector phase of pulmonary DTH reactions. Studies on cutaneous DTH responses, however, were unable to show an effect of cimetidine during the elicitation phase (111, 160, 229, 266) which is in agreement with the cutaneous results obtained in this thesis. In contrast, extensive pretreatment with cimetidine before the onset of sensitization has been reported to cause an enhanced

cutaneous DTH response (13). It was speculated that this action of cimetidine promoted the inhibition of a suppressor factor produced by T lymphocytes. However, these interpretations are of little significance to the experiments of this thesis because the cimetidine treatment during the challenge with TDI was shorter and the used dosage was less when compared with the published studies. Data from this thesis led to the hypothesis that histamine is released (from mast cells or other sources?) after intranasal challenge with TDI and that it subsequently activates H_2 receptors on immune cells (i.e. lymphocytes, mast cells, granulocytes), thus modulating the secretion of bioactive mediators. In conclusion, in chapter 5 it was demonstrated that skin sensitization with TDI caused a marked mucosal mast cell activation. Although, in W/W^v mice tracheal hyperreactivity was still evident, it is still possible that mast cell mediators play a crucial role in the development of TDI-induced tracheal hyperreactivity.

In the last two experimental chapters of this thesis we have attempted to further unravel the DTH-like mechanism of TDI-induced occupational asthma. In chapter 6 the role of T lymphocytes was studied. In athymic nude BALB/c mice no tracheal hyperreactivity after TDI-sensitization and challenge could be detected, indicating that T lymphocytes are involved in the development of tracheal hyperreactivity. Furthermore, the contribution of $CD4^+$ and $CD8^+$ T lymphocytes during the effector phase of TDI-induced DTH-like reactions was investigated using *in vivo* depletion of the specific T cell subsets. A depletion of approximately 80% in the peripheral blood and lymphoid tissue of $CD8^+$ T lymphocytes of TDI-sensitized mice completely inhibited the TDI-induced tracheal hyperreactivity. In comparison, depletion of approximately 75% of $CD4^+$ T lymphocytes resulted in partial inhibition of the TDI-induced tracheal hyperreactivity. These results indicated that not only $CD4^+$ T cells, which are the classical DTH effector T cells, but that in addition $CD8^+$ T cells contribute to TDI-induced tracheal hyperreactivity.

$CD8^+$ T cells can be divided into $CD8^+$ Tc1 cells and $CD8^+$ Tc2 cells on the basis of their cytokine release profile. Through the release of Tc1-type cytokines (IFN–γ) or Tc2-type cytokines (IFN–γ, IL-4, IL-5 and IL-10) $CD8^+$ T lymphocytes can influence Th1 or Th2 responses (142). Recently, in DNFB and oxazolone-induced contact hypersensitivity (CHS) it was shown that depletion of $CD8^+$ T cells prior to sensitization resulted in a complete abolition of the ear swelling response 24 hr after the challenge, whereas *in vivo* $CD4^+$ T cell depletion caused an increased and prolonged CHS response (320). Moreover, it was demonstrated that the CHS effector $CD8^+$ T cells isolated from lymph nodes produced IFN–γ and the negatively regulating $CD4^+$ T cells produced predominantly IL-4 and IL-10 (320). In agreement with the finding in this thesis that $CD8^+$ T cells are involved

in TDI-induced hyperreactivity, an increase in circulating $CD8^+$ T lymphocytes was associated with TDI-exposed subjects who exhibited the late phase asthmatic reaction (83, 163). Additionally, the majority of T cell clones, derived from the bronchial mucosa of two patients with TDI-induced occupational asthma, were $CD8^+$ producing IFN–γ and IL-5 in response to nonspecific stimuli (69). In summary, in TDI-induced DTH-like pulmon changes both $CD4^+$ T cells and $CD8^+$ T cells appear to play a role as DTH effector T cells.

Finally, in chapter 7 a TDI-specific lymphocyte factor was isolated. Within 1 to 2 days after sensitization DTH-initiating lymphocytes ($Thy\text{-}1^+$, $Lyt\text{-}1^+$, $CD4^-$, $CD8^-$, $IL\text{-}2R^-$, $IL\text{-}3R^-$ and $B220^+$ (115)) produce a hapten-specific lymphocyte factor. The TDI-specific lymphocyte factor was isolated from the supernatant of 2 days cultured spleen cells isolated from TDI-sensitized mice and purified by hapten-affinity chromatography. In further experiments, the biological activity of the TDI-specific lymphocyte factor was examined. Passive sensitization by intravenous injection of the TDI-specific lymphocyte factor followed after 30 minutes by a topical or intranasal challenge with TDI caused a significant ear swelling, mast cell activation and *in vitro* tracheal hyperreactivity 2 hr after the challenge. The increased ear swelling in TDI-factor sensitized mice was not found 2 hr after topical challenge with DNFB, indicating that the TDI-factor was hapten-specific. Furthermore, the isolation procedures used (denaturating conditions) exclude the possibility that IgE was isolated. It is interesting to note the similarities between TDI-factor-induced responses and the active model discussed in chapter 2. However, certain discrepancies arise which deserve an explanation.

In DTH reactions, hapten-specific lymphocyte factors have been demonstrated to be responsible for the early phase reactions. Indeed, the TDI-specific lymphocyte factor isolated in this thesis elicited numerous responses 2 hr after the challenge, which is the time point that others have observed early responses (4, 307). Why then has an early response never been observed in our active short exposure model (discussed in chapter 2)? In chapter 7, it has been suggested that the choice of anaesthesia could be responsible for this phenomenon. It was demonstrated that the cutaneous ear swelling response in TDI-factor sensitized mice was absent when sodium pentobarbitone anaesthesia was used. Previously, all other experiments in this thesis were performed using sodium pentobarbitone and possibly, using light ether or other anaesthesia may lead to the detection of an early phase in TDI-induced DTH-like responses. In contrast to the active sensitization protocol, passive sensitization with a TDI-specific lymphocyte factor did not cause an increased ear swelling response 24 hr after the challenge. This discrepancy is reasonable because the later response (24-48 hr) in DTH reactions are induced by DTH

effector T cells (discussed in chapter 6) which are unrelated to the cells that produce the TDI-specific lymphocyte factor.

Interestingly, in the final experiments of this thesis it was demonstrated that the TDI-specific lymphocyte factor was able to induce a direct *in vivo* bronchoconstriction 2.5 minutes after the challenge. In general, an immediate bronchoconstriction has been associated with IgE-dependent mechanisms. For example, both in subjects with atopic asthma as well as in several animal models for atopic asthma, direct bronchoconstrictions have been detected. In addition, in guinea pig models for IgE-mediated TDI-induced occupational asthma, TDI-exposure was shown to cause a direct bronchoconstriction. However, in animal models for IgE-independent DTH-like occupational asthma immediate bronchoconstriction has never been reported and it is an interesting hypothesis that the direct bronchoconstriction observed in subjects with IgE-independent occupational asthma is caused by a hapten-specific lymphocyte factor. Future experiments will focus on the presence of direct *in vivo* bronchoconstriction in TDI-sensitized mice after the active sensitization protocols (both long and short exposure) described in this thesis in chapter 2 and in chapter 3. Furthermore, the isolation of a TDI-specific lymphocyte factor, which to date has only been isolated in the mouse, has implications for TDI-induced occupational asthma in man. Serological screening of TDI-exposed workers not only for TDI-specific IgE antibodies but in addition for a TDI-specific lymphocyte factor may lead to an early and more thorough detection of sensitized workers.

One feature that has not been covered in this thesis is the role for B lymphocytes in TDI-induced DTH-like responses. The TDI-specific lymphocyte factor is produced by DTH-initiating lymphocytes, which can be either T or B cells. The research on the effector phase of the DTH responses focused on the role of $CD4^+$ and $CD8^+$ T lymphocytes and does not negate a role for B lymphocytes in the initiation of the reaction. Although this subject was not addressed in this thesis it would be fascinating to elucidate the role of B cells in pulmonary DTH-like reactions.

Cutaneous responses

Classically, DTH responses are measured in the skin. In this thesis in addition to the pulmonary responses, the cutaneous responses to TDI were also determined. They were routinely followed in most experiments in this thesis primarily as a check for successful TDI-sensitization which was achieved via the cutaneous route. Although in some cases the cutaneous responses were in agreement with the pulmonary responses, striking differences were often observed. First, with respect to the T cell dependency, TDI-induced ear

swelling responses, like pulmonary responses, were absent in athymic nude mice. However, in contrast to the pulmonary responses, adoptive transfer of TDI-sensitized lymphoid cells to naive recipients did not lead to an increased ear swelling 24 hr after the challenge. Furthermore, *in vivo* depletion of $CD4^+$ and $CD8^+$ T lymphocytes during the effector phase had no effect on the TDI-induced ear swelling response. Several explanations can be given for these discrepancies. With respect to the adoptive transfer studies, these were experiments which were optimized (number of transferred cells, time of challenge) to acquire TDI-induced tracheal hyperreactivity. Changes in experimental parameters (i.e. different amounts of transferred cells) might have led to a transferred ear swelling response, but these options were not explored. With respect to the *in vivo* depletion of $CD4^+$ and $CD8^+$ T lymphocytes, a depletion of 100% was never accomplished. Depletion of approximately 80% of the $CD4^+$ and $CD8^+$ T lymphocytes was sufficient to inhibit the TDI-induced tracheal hyperreactivity but apparently not to exert an effect on TDI-induced ear swelling. Furthermore, the depletion was assessed in the peripheral blood and lymphoid tissue and there might have been different depletions locally in the air ways and skin.

The modulations of TDI-induced responses by sensory neuropeptides (chapter 4) and mast cell mediator antagonists (chapter 5) all failed to exert an effect on TDI-induced ear swelling, whereas TDI-induced tracheal hyperreactivity was markedly affected. For example, neither capsaicin pretreatment nor blockade of the NK_1 receptor prevented the development of TDI-induced ear swelling. Moreover, both ketanserin and cimetidine were unable to block the TDI-induced cutaneous response while the pulmonary responses were attenuated. These results illustrate that the TDI-induced cutaneous responses are probably regulated through a different mechanism than the pulmonary response. Interestingly, similar discrepancies were obtained in the DNFB-induced DTH-like reactions. DNFB-induced mucosal exudation and leukocyte accumulation in the mouse airways were inhibited by NK_1 receptor blockade whereas the cutaneous response was unaffected. An additional explanation for the discrepancy between lung and cutaneous DTH reactions could be that the TDI-induced cutaneous response was extremely aggressive and therefore difficult to manipulate. It must be reiterated however, that the experiments in this thesis focused on the pulmonary responses, therefore the conditions for cutaneous DTH reactions might not have been optimal.

Conclusion

Altogether, the data presented in this thesis have indicated that TDI causes occupational asthma through different immunological mechanisms. In *figure 1* a hypothetical scheme for the TDI-induced IgE-independent and IgE-mediated mechanism is depicted. It is highly likely that a close interaction and a fine balance exists between both mechanisms. It will be difficult to predict which reaction occurs in which subject and additional research is required both in animal models and in exposed workers suffering from TDI-induced asthma. Until the mechanism of action of TDI-induced occupational asthma are fully eliminated and therapies discovered we must rely on detecting occupational asthma and removing sensitized workers from the workplace. This point was addressed in this thesis and a possible solution has been found. It is hopeful that the presence of TDI-specific lymphocyte factors in man could aid screening of exposed workers and the detection of those at most risk developing occupational asthma.

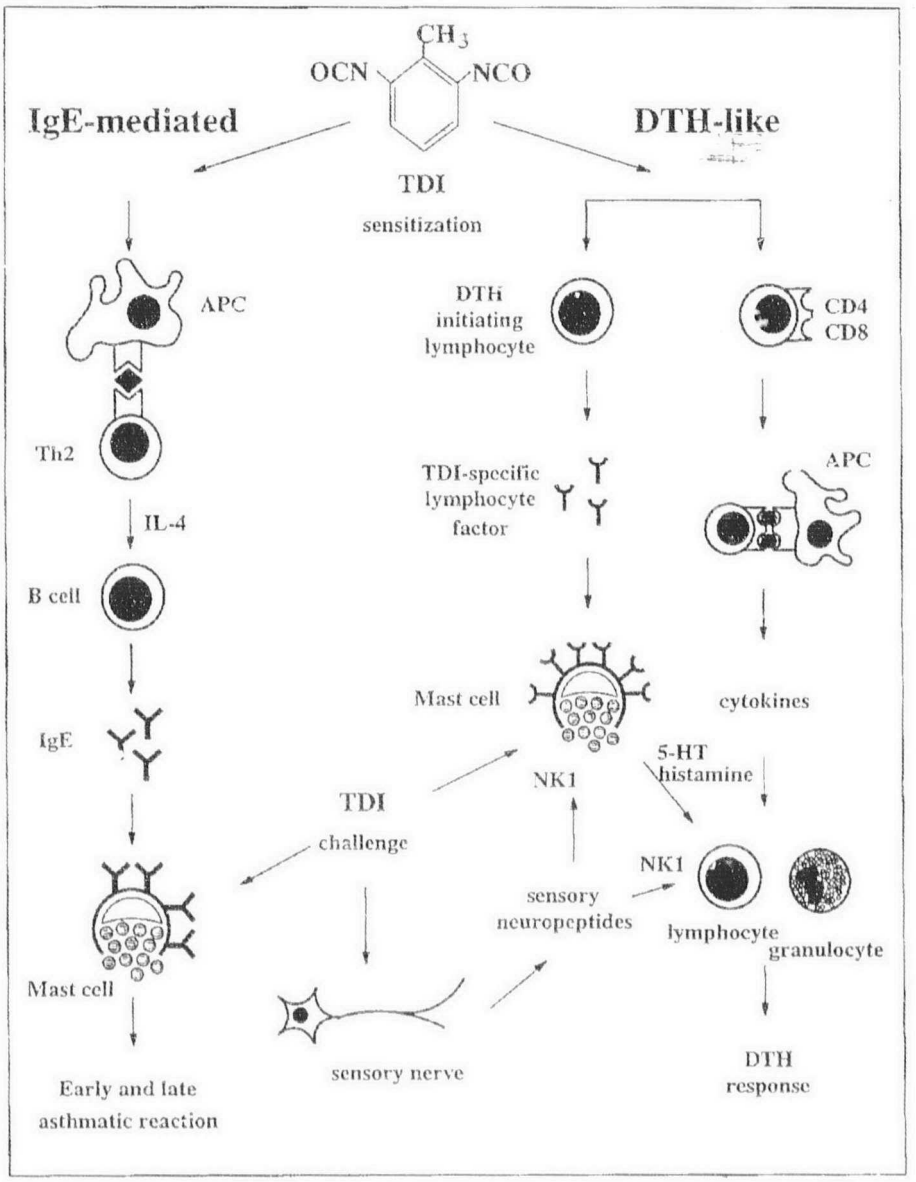

Figure 1 Hypothetical scheme of the mechanisms of action of TDI.

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Samenvatting

Beroepsastma is een steeds groter wordend probleem in de ontwikkelde landen. Tot nu toe zijn al meer dan tweehonderd oorzaken van beroepsastma bekend en verwacht wordt dat dat aantal in de toekomst zal toenemen. De stoffen die beroepsastma veroorzaken kunnen worden verdeeld in twee groepen: 1) allergenen met een hoog molecuul gewicht en 2) stoffen met een laag molecuul gewicht die aan lichaamseigen eiwitten moeten binden om antigeen te worden. De stoffen met een hoog molecuul gewicht, zoals eiwitten uit meel, dierlijke allergenen en enzymen, veroorzaken astma via een allergisch mechanisme, waarbij IgE antistoffen gevormd worden. De laag moleculaire verbindingen, zoals isocyanaten, stofdeeltjes afkomstig uit hout en amines, vormen de grootste groep veroorzakers van beroepsastma. Het is onbekend via welk mechanisme deze stoffen beroepsastma veroorzaken. Eén van de meest opvallende verschillen met allergisch, IgE-afhankelijk astma is dat slechts in een klein deel van de patiënten een verhoging wordt gevonden in IgE antistoffen. De grootte van deze groep is afhankelijk van de stof die beroepsastma veroorzaakt. Zo worden bij slechts 20% van de patiënten met isocyanaat-geïnduceerde astma IgE antistoffen in het serum gevonden, terwijl voor amine-geïnduceerde astma geldt dat bij 50% van de patiënten IgE-antistoffen worden gevonden. Deze tweedeling van patiënten met beroepsastma geïnduceerd door een laag moleculaire verbinding (IgE-afhankelijk en IgE-onafhankelijk), is een aanwijzing dat tenminste twee immunologische mechanismen van belang zijn bij het ontstaan van deze vorm van beroepsastma.

Isocyanaten vormen de grootste groep van de laag moleculaire verbindingen die beroepsastma veroorzaken. Vooral tolueendiisocyanaat (TDI) wordt veelvuldig gebruikt in de industrie, met name in de produktie van verf en polyurethaanschuim (isolatiemateriaal). Ongeveer 5-10% van de mensen die met TDI werken, ontwikkelt beroepsastma. Bij deze mensen wordt een niet-specifieke hyperreaktiviteit (overgevoeligheid voor prikkels van buitenaf) en ontsteking van de luchtwegen waargenomen, die wordt gekarakteriseerd door een toename in T cellen, neutrofiele- en eosinofiele granulocyten en mestcellen. Personen die met TDI in aanraking komen, worden getest op de aanwezigheid van TDI-specifieke IgE antistoffen. Helaas wordt met deze procedure slechts 20% van de patiënten met beroepsastma geïdentificeerd als overgevoelig voor TDI. Het grootste deel van de patiënten wordt op deze manier gekarakteriseerd als 'TDI-ongevoelig' en zal blijven werken met TDI, wat uiteindelijk zal resulteren in permanente beschadiging van de

luchtwegen en astma. Het is daarom uiterst belangrijk het werkingsmechanisme van TDI-geïnduceerd beroepsastma te onderzoeken. Het eerste doel van dit promotieonderzoek is om twee modellen in de muis te ontwikkelen die gebruikt kunnen worden om de effecten van TDI te bestuderen (een IgE-afhankelijk en een IgE-onafhankelijk model). De tot nu toe beschreven caviamodellen richten zich volledig op het IgE-afhankelijke mechanisme van TDI-astma. In dit proefschrift ligt de nadruk van het onderzoek vooral op het IgE-onafhankelijke mechanisme van TDI-astma.

Allereerst beschrijft hoofdstuk 2 de ontwikkeling van het IgE-onafhankelijk model in de muis voor TDI-astma. Verschillende behandelingen met TDI werden geprobeerd om de muizen "TDI-astmatisch" te maken. De muizen werden voor de eerste keer blootgesteld aan TDI (sensibilisatie) op de geschoren buikhuid en de vier poten (op dag 0 en dag 1, twee keer per dag). Vervolgens werden de longen van de muizen blootgesteld aan TDI (intranasale challenge) met 1% TDI op dag 8. Door deze behandeling kregen de muizen de meeste kenmerken van humane TDI-astma: 1) geen produktie van TDI-specifieke IgE antistoffen, 2) *in vitro* hyperreaktiviteit (overgevoeligheid) van de trachea (luchtpijp) 24 uur na de challenge en 3) verhoogde myeloperoxidase (MPO) aktiviteit in de longen en in het serum. MPO is een enzym dat voorkomt in de granules van neutrofiele granulocyten, en de toename in MPO aktiviteit duidt op een aktivatie van deze cellen.

In hoofstuk 3 is aangetoond dat blootstelling aan TDI ook kan leiden tot de produktie van IgE antistoffen in de muis. Door de blootstelling (sensibilisatie) aan TDI te verlengen van twee keer in één week naar één keer per week gedurende zes achtereenvolgende weken werden TDI-specifieke IgE antistoffen gevormd. Bovendien leidde langdurige blootstelling aan TDI tot een *in vitro* en *in vivo* hyperreaktiviteit van de luchtwegen 3 uur na de challenge. De verschillen tussen het korte blootstellingsmodel en het lange blootstellingsmodel komen dus niet alleen tot uiting in de aan- of afwezigheid van IgE antistoffen, maar ook in het tijdstip van het optreden van hyperreaktiviteit na de challenge. Of de hyperreaktiviteit van de luchtwegen in het lange blootstellingsmodel inderdaad veroorzaakt wordt door de aanwezigheid van TDI-specifieke IgE antistoffen moet nog blijken uit toekomstige experimenten.

Het korte- en het lange blootstellingsmodel (hoofdstuk 2 en 3) kunnen beide worden gebruikt om TDI-astma te onderzoeken. De volgende hoofdstukken in dit proefschrift wijden zich echter volledig aan het korte (IgE-onafhankelijke) blootstellingsmodel, omdat bij 80% van de patiënten met TDI-astma geen IgE antistoffen worden gevonden en omdat de bestaande modellen vooral de IgE-afhankelijke reaktie van TDI bestuderen.

De hypothese is dat IgE-onafhankelijke TDI-astma wordt veroorzaakt door een cel-gemedieerde, vertraagd-type overgevoeligheids (VTO) reaktie. VTO reakties worden voornamelijk onderzocht in de huid en makkelijk opgewekt door laag moleculaire verbindingen. De cellulaire reakties die plaatsvinden tijdens een VTO reaktie staan schematisch weergegeven in figuur 3 van hoofdstuk 1. Tijdens de eerste, sensibilisatie fase bindt de stof (het hapteen) aan lichaamseigen eiwitten en worden VTO-initiërende lymfocyten geaktiveerd tot de produktie van hapteen-specifieke lymfocytfactoren. Vervolgens binden deze lymfocytfactoren aan mestcellen en eventueel aan andere cellen van het immuunsysteem. Na een tweede blootstelling aan het antigeen (de challenge fase) bindt het antigeen aan de antigeen-specifieke lymfocytfactoren gebonden aan mestcellen. Hierdoor worden de mestcellen geaktiveerd wat resulteert in de vrijzetting van mediatoren, zoals serotonine. Serotonine zorgt voor een verhoogde vaatdoorlaatbaarheid waardoor VTO effector T cellen uit de bloedcirculatie in het weefsel kunnen infiltreren. Deze VTO effector T cellen herkennen het antigeen dat gepresenteerd wordt door antigeenpresenterende cellen (APC). De VTO effector T cellen worden hierdoor aangezet tot de produktie van biologisch aktieve stoffen (cytokines), die op hun beurt verantwoordelijk zijn voor de VTO reaktie.

Het tweede doel van dit proefschrift is om te onderzoeken of de TDI-geïnduceerde effecten in het korte blootstellingsmodel voor TDI-astma (IgE-onafhankelijk) worden veroorzaakt door VTO reakties. De rol van de verschillende celtypen die van belang zijn bij VTO reakties (mestcellen (hoofdstuk 5), T cellen (hoofdstuk 6) en TDI-specifieke lymfocytfactoren (hoofdstuk 7)) in TDI-geïnduceerde hyperreaktiviteit van de luchtwegen in de muis is onderzocht. Al deze celtypen kunnen worden gereguleerd door sensorische neuropeptiden. Sensorische neuropeptiden liggen opgeslagen in sensorische zenuwen, die te vinden zijn in de gehele luchtwegen en in nauw contact staan met de verschillende cellen van het immuunsysteem. In hoofdtuk 4 is het effect van TDI op de sensorische zenuwen in de luchtwegen van de muis onderzocht. TDI veroorzaakte *in vitro* een relaxatie van de trachea, die volledig geremd werd door preïncubatie met de neurokinine-$_1$ (NK_1) receptor antagonist, RP67580. De conclusie is dat TDI sensorische neuropeptiden (b.v. substance P of neurokinine A) uit de sensorische zenuwen vrijmaakt die via activatie van de NK_1-receptor een relaxatie van de luchtwegen teweeg brengen. Vervolgens is onderzocht of deze activatie van de sensorische zenuwen ook *in vivo* optreedt en een rol speelt in het ontstaan van de TDI-geïnduceerde tracheale hyperreaktiviteit. Behandeling van de muizen met capsaicine (wat resulteert in de volledige depletie van sensorische neuropeptiden) voor sensibilisatie en challenge met TDI, resulteerde in een volledige remming van de

hyperreaktiviteit van de trachea 24 uur na de challenge. De TDI-geïnduceerde hyperreaktiviteit werd ook geremd door behandeling met de NK_1-receptor antagonist RP 67580, wanneer deze voor de challenge intraveneus werd toegediend. Samenvattend kan gesteld worden dat sensorische neuropeptiden van belang zijn voor het ontstaan van de hyperreaktiviteit tijdens de challenge fase. In de luchtwegen van de muis veroorzaken sensorische neuropeptiden direct een relaxatie. De hyperreaktiviteit van de trachea wordt waarschijnlijk veroorzaakt door een interaktie van neuropeptiden met cellen van het immuunsysteem, zoals lymfocyten, mestcellen, macrofagen en neutrofiele granulocyten.

In hoofdstuk 5 is de rol van de mestcellen in TDI-geïnduceerde hyperreaktiviteit van de trachea onderzocht. Ten eerste leidde TDI sensibilisatie tot mestcel activatie, wat gemeten werd als verhoging van een protease dat specifiek is voor mucosale mestcellen (MMCP-1 in de muis) in het bloed en gehomogeniseerd longweefsel. Vervolgens werd onderzocht of deze mestcel aktivatie belangrijk was voor het ontstaan van de hyperreaktiviteit. Hiervoor werden twee benaderingen gebruikt. Ten eerste werd gekeken of de hyperreaktiviteit nog aanwezig was na TDI sensibilisatie en challenge in muizen die door een genetisch defect geen mestcellen hebben. Deze mestcel deficiënte muizen ontwikkelden echter nog steeds een tracheale hyperreaktiviteit 24 uur na de challenge. De tweede benadering was om de rol van de mestcelmediatoren histamine- en serotonine, die vrijkomen tijdens de VTO reaktie, te onderzoeken. De TDI-geïnduceerde hyperreaktiviteit werd volledig geremd door behandeling van de muizen met histamine en serotonine receptor antagonisten (respectievelijk cimetidine en ketanserine). Concluderend kunnen we stellen dat zowel histamine als serotonine van belang zijn voor het ontstaan van de hyperreaktiviteit. Er zijn echter ook andere bronnen van deze mediatoren dan de mestcel, dus de rol van de mestcel zal nog moeten worden aangetoond.

Hoofdstuk 6 beschrijft de rol van de T cellen in de VTO reaktie geïnduceerd door TDI in de luchtwegen van de muis. Ten eerste trad er geen hyperreaktiviteit van de trachea op in muizen zonder T cellen (athymische naakte muizen). Ten tweede werd de hyperreaktiviteit geremd wanneer in TDI-gesensibiliseerde muizen de T cellen (zowel $CD4^+$ als $CD8^+$) werden gedepleteerd door toediening van anti-CD4 en anti-CD8 antistoffen. Ten derde werden cellen geïsoleerd uit milt en lymfklieren van TDI-gesensibiliseerde muizen. Nadat deze celpopulatie werd ingespoten in onbehandelde muizen, bleken deze ontvangermuizen ook hyperreaktiviteit van de trachea te vertonen 24 uur na de challenge. Deze experimenten hebben duidelijk aangetoond dat T cellen een belangrijke rol spelen in het ontstaan van de hyperreaktiviteit van de trachea in TDI behandelde muizen.

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In hoofdstuk 7 is beschreven dat de TDI-specifieke lymfocytfactor (TDI-factor) is geïsoleerd uit miltcellen van TDI-gesensibiliseerde muizen. Vervolgens werd de biologische aktiviteit van deze TDI-factor aangetoond Het bleek mogelijk door intraveneuze toediening met de TDI-factor muizen te sensibiliseren. Drie uur na intranasale challenge met TDI werd bij met TDI-factor behandelde muizen tracheale hyperreaktiviteit en mestcelaktivatie gemeten. Hieruit blijkt dat er inderdaad een TDI-specifieke lymfocytfactor wordt geproduceerd na TDI sensibilisatie die in staat is hyperreaktiviteit en mestcelaktivatie te veroorzaken. Nog interessanter was de waarneming dat bij TDI-factor gesensibiliseerde muizen een directe *in vivo* bronchoconstrictie optrad. Dit zou kunnen betekenen dat bij patiënten met IgE-onafhankelijke beroepsastma een specifieke lymfocytfactor verantwoordelijk is voor de bronchoconstrictie die bij deze patiënten optreedt.

Samenvattend zijn in dit proefschrift twee modellen beschreven in de muis om TDI-geïnduceerde beroepsastma te onderzoeken. Het IgE-onafhankelijke mechanisme van TDI-astma verloopt waarschijnlijk via een VTO reaktie, waarin lymfocytfactoren een zeer belangrijke rol spelen. Om te voorkomen dat steeds meer mensen beroepsastma krijgen, zouden mensen die met TDI in aanraking komen getest moeten worden op de aanwezigheid van zowel een TDI-specifieke lymfocytfactor als op de aanwezigheid van TDI-specifieke IgE antistoffen. Op die manier zouden meer mensen worden herkend als 'TDI gevoelig' en kunnen maatregelen worden genomen om ergere schade (astma) te voorkomen.

Dankwoord

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Curriculum vitae

De auteur van dit proefschrift werd op 11 december 1969 geboren te Amsterdam. Na het behalen van het Gymnasium–β diploma aan het Waterlant College te Amsterdam, werd in 1988 begonnen met de studie Farmacochemie aan de Vrije Universiteit te Amsterdam. De doctoraal fase bestond uit het hoofdvak moleculaire toxicologie (Dr A.R. Goeptar, Prof.dr N.P.E. Vermeulen) en het bijvak organische synthese van biologisch aktieve verbindingen (Dr R.C. Vollinga, Prof.dr H. Timmerman). Het doctoraal examen werd behaald in maart 1993. In april 1993 werd begonnen met een promotieonderzoek bij de afdeling Farmacologie en Pathofysiologie van de Universiteit Utrecht in samenwerking met het Laboratorium voor Pathologie en Immunobiologie van het Rijks Instituut voor Volksgezondheid en Milieu. Het promotieonderzoek, waarvan de resultaten beschreven staan in dit proefschrift, is uitgevoerd onder begeleiding van Dr Theresa L. Buckley, Dr Henk Van Loveren en Prof.dr Frans P. Nijkamp.

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CERTIFICATE OF AUTHENTICITY

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